

# PHYSIOLOGY OF FUNGI

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## PREFACE

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FUNGAL PHYSIOLOGY as an independent discipline is comparatively of recent origin. During the last three decades the subject has made rapid strides and at the same time has contributed substantially to other sciences like biochemistry, genetics and microbiology. Despite its practical utility the subject is still on the periphery so far as the curricular programmes of the Indian Universities are concerned. Lack of proper reading material has been possibly one of the major factors for this situation. Although we were seriously concerned about it since long, the opportunity arose only when the University Grants Commission sanctioned us a book writing project.

This text is designed for post-graduate and research students of fungal physiology. Important physiological processes of fungi like respiration, growth and metabolism are fully elucidated with a biochemical framework. Hormonal regulation of sex in fungi forms the basis of chapter on Physiology of Reproduction. A detailed account of a wide variety of metabolic products of fungi is also incorporated to cater the needs of students of applied biology and agriculture. The text is liberally laced with recent references which open the doors to many interesting research problems.

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have given inspiring comments on this presentation, we are grateful to them.

Inevitably the text must include errors and omissions for which we own undiluted responsibility. Any comments or suggestions for future amendments will be gratefully acknowledged.

K. S. BILGRAMI  
R. N. VERMA

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## INTRODUCTION

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Fungi have an ancient lineage and are believed to have existed in devonian and pre-cambrian era. Their impact on man's destiny was recognised early in the human civilization. Fungal diseases find reference in Vedas (1200 B.C.) and Bible. Romans were fully aware with the problem of rust diseases of cereals. They were also familiar with the food and medicinal values of fungi. First record of myco-toxicoses dates back to 450-456 B.C. Use of fungi in preparation beverages was quite prevalent in ancient times.

Scientific knowledge of fungi, however, had to wait till the dawn of microscopy, this was obviously due to the fact that majority of them are invisible to unaided eyes. The advent of De Bary towards the middle of the nineteenth century opened the gates for Mycology to move towards right direction and with quicker pace. The advancing frontiers of fungal knowledge during the last fifty years have amply demonstrated that these ubiquitous organisms exert a decisive influence on human affairs in various ways.

However, to a layman of the present century fungi continue to be mere scavengers, disposing off dead organic remains of plants and animals. Generally people are familiar with macroscopic agarics growing in forests and fields, leathery bracket-fungi hanging on tree-bran-ches or decaying logs; puff-balls appearing in the pastures; etc. but they are unaware of the microscopic fungi beneath the soil and their significant role in agriculture. Even now the common man is hardly conversant with the fungi being exploited in the industries for the synthesis of a large variety of organic compounds, drugs and vitamins. That several antibiotics are the products of fungal activities is a know-ledge which remains confined to a section of intelligentia alone. On academic level also, fungi have aroused keen interest. Being simpler and adaptive, they are ideal tools of research and have greatly

helped to unravel the mystery of various biological phenomena. With so much to contribute to the applied as well as the academic field, fungi and their study can no more be kept confined to the mycologist alone, rather their knowledge should be allowed to disseminate down to the common man on the street. The more we understand their life and physiology, the better we can utilize them in agriculture, industry and medicine, and at the same time the greater will be our control over their harmful activities as pathogens of plants, animals and man, and as destroyer of timber, textile, food and feed.

### SPECIAL FEATURES OF FUNGI

Fungi are a diverse group of organisms and have interesting characteristics. Although they have been conventionally treated as plants without root, stem and leaves, they are like animals, devoid of chlorophyll and hence nutritional heterotrophs. The versatile taxonomist Linnaeus erred with them and placed a few fungal specimens collected by him among the *Vermes* (Worms). In fact a separate Kingdom status has been proposed for fungi in a new classification system, wherein four kingdoms have been recognised in place of the traditional two (Whittaker, 1959), including *Protista* (unicellular organisms), *Plantae* (multicellular plants), *Fungi* and *Animalia* (multicellular animals). It will be seen from the discussion in the coming chapters that fungi have something common with one group and some with the others, maintaining thereby an overall individuality. This is true even at the cellular level; for example a fungal cell resembles a plant and animal cell in respect of its true nucleus, but with regard to cell-wall it differs from an animal cell, and resembles a plant cell. In the absence of chlorophyll, however, a fungal cell is closer to its animal counterpart. An outline of the characteristics of a fungal cell, which as we know is the functional unit of an organism, will further illustrate the point.

### CELLULAR CHARACTERISTICS OF FUNGI

Apart from some important differences, the basic structure of a fungal cell is similar to those of other Eukaryotes as well as of higher plants and animals. They have (i) their nuclear material surrounded by a nuclear envelop, and (ii) their cell-protoplast is better organized and compartmentalized than those of the Prokaryotes. Among themselves, of course fungi exhibit marked variations with regard to one



or the other cell component, and hence there is no rigid concept regarding the constitution of a fungal cell. Structure and ultrastructure of fungal cells have been adequately described in some of the reviews (Raper and Esser, 1964; Moore, 1965; Bracker, 1967), and only the distinguishing features and the functional aspects of different cell components need to be emphasized here.

### Nuclei

The earlier controversy regarding fungal nuclei obviously due to their small size, has been resolved with the help of electron microscopic studies. It is now established that fungal nuclei possess a nuclear envelop (Robinow and Bakerspigel, 1965; Hawker, 1966). The nuclear envelop is characterized by the presence of gaps or pores, which in some cases may remain scattered all round but in others they are restricted to a specific region. These pores are considered to help in facilitating exchange of substances between nuclei and the cytoplasm (Fawcett, 1966). Another distinguishing feature of the nuclear envelop of fungal nuclei is their persisting nature and they do not usually disintegrate during nuclear division (Berlin and Bowen, 1965; Fuller and Reichle, 1965; Hawker, 1965; Motta, 1967), rather they constrict in the middle at the time of formation of daughter nuclei. Even during some nuclear-fusion phenomena, *e.g.*, during conjugation of yeast cells (Conti and Brock, 1965) or that occurring in basidia (Wells, 1965) the nuclear envelops maintain their integrity.

Similar is the behaviour of nucleolus during somatic nuclear division in most of the fungi. Of course, it may not be distinguishable in all fungal nuclei, as in many cases the nucleoplasm appears to be homogenous. During reduction division, however, both nucleolus and the nuclear envelop disappear towards the end of prophase.

Chromosomes and chromatin are hardly distinct in fungal nuclei, and even during division they are very small and patchy. Another characteristic is their random attachment to the metaphase spindle without forming an equatorial plate. This is true both for somatic as well as reduction divisions of the nucleus, although during the latter case they may sometime assemble at the equator. Thus, it may be noted that the somatic nuclear division in fungi exhibits more deviation from the normal mitotic division of the higher organisms. Moore (1964, 1965) prefers to designate fungal mitosis as *Karyochorisis* chiefly on the ground that the persisting nuclear envelop invaginates to produce two daughter nuclei.

Other components functioning in the nuclear division have been



described in several fungi. The spindle apparatus along with microtubules recorded in several fungi are characteristically intranuclear (Moore, 1964; Rabinow and Bakerspigel, 1965; Moore 1966; Motta, 1967). True centrioles have been observed in a number of fungi, particularly those with motile stage (Berlin and Bowen, 1964, 1965; Fuller, 1966; Lessie, 1967; Reichle and Fuller, 1967). In this respect, fungi exhibit similarity to animal cells. In fact, the centrioles of fungal sporangia, vegetative hyphae and zoospores have been found to be similar in composition to those of the animal cells (Fawcett, 1966). There are some interesting observations regarding fungal centriole as well as spindle apparatus. As for instance, in some fungi a true centriole is present outside the nuclear envelop, and the intranuclear spindle terminates just inside the envelop without any apparent connection with the centriole. In other fungi like *Armillaria mellea*, the centriole-like body does not have a typical composition of a true centriole, but it is attached to the spindle fibres, which penetrate through the discontinuous nuclear envelop at the poles (Motta, 1967). In some fungi the nuclear division is designated as astral because in them some additional fibrils radiate from the centriole like a sunburst, producing astral ray configuration. In all these aspects fungal nuclear division differs from a typical mitosis of higher plants, which is characteristically extranuclear, acentric (without a centriole) and anastral (without aster); and is more akin to that of the higher animals, which is extranuclear but centric and astral.

In fungi, nuclear division in a vegetative cell is seldom followed by a cytoplasmic division. Therefore, the fungal body remains as a continuous mass, which grow in size and increase in the number of nuclei by nuclear division only. In same higher fungi, septa do develop, but they are incomplete leaving pores through which the protoplasm may continue to flow. True cells complete with nucleus and cell-boundary are produced only during reproduction.

#### **Other Cell Organelles and Cell Inclusions**

Most of the cell-organelles found in higher organisms are found in fungal cells also, including mitochondria, endoplasmic reticulum, ribosomes, golgi apparatus etc. Some specific components like loma-some, various kinds of microtubules etc. have also been found in some fungi but plastids are the notable absentees.

(a) *Mitochondria*. Mitochondria found in fungi may have different forms and number in different species, at different stages of growth, and under varying external conditions. Functionally, they are com-

parable to those of higher plants and animals (Zalokar, 1965; Fawcett, 1966). Containing various respiratory enzymes, they are the site of energy-producing reactions of respiration. Fungal mitochondria contain a DNA component (Luck and Reich, 1964; Schuster, 1965) which shows that they are partially autonomous.

(b) *Endoplasmic reticulum and ribosomes*. The fungal endoplasmic reticulum (ER) may be considered as the most pleomorphic component of the fungal cell. A variety of membranous components with varying forms and functions has been treated as ER, and their real significance in the physiology of fungi could not be clearly ascertained. Recently, ER has been assigned an important role in the tip growth and hyphal extension of fungi (Grove *et al.*, 1970), as has been discussed in a chapter on growth. In fact, it has been suggested that the lipid and protein required for the continued synthesis of plasma membrane are biosynthesized in the granular ER. Granular or the rough ER with attached ribosome particles have been recorded in a number of fungi (Gay and Greenwood, 1966; Lessie, 1967) but presence of the smooth ER of the type found in animals is yet to be ascertained. Most of the fungal ribosomes are found free in the cytoplasm as fine granules, like those of any other cell. Free ribosomes are thought to synthesize and replenish those cellular proteins which control cellular activities.

Like ribosomes, certain other cell-organelles, including plasma membrane and mitochondria, remain associated with ER, which shows that they may have functional relationship too.

(c) *Golgi apparatus*. Golgi apparatus (GA) generally consists of several interrelated dictyosomes, where each dictyosome represents a stack of smooth membraned cisternae. Dictyosomes which have been recorded in a limited number of fungi, particularly in Phycomycetes are generally associated with ER or nuclear envelop. Recent studies (Grove *et al.*, 1970) on fungal tip growth have shown that dictyosomes are the logical site for transformation of the membrane from the ER type to plasmalemma type, which is essential for the continued synthesis of the plasma membrane. Details have been discussed in the chapter on "Growth."

(d) *Lomasomes*. These membranous structures are found in a matrix between the plasmalemma and cell-wall, and therefore have been named as Lomasomes (= border bodies). Although they have been claimed in a good number of fungi, lack of any definite information regarding their functions, genesis, etc. have led to some rethinking, whether they are actually present in a living fungal cell or

they simply arise as a traumatic response to fixing agents. Nevertheless, various roles have been suggested for them; a few important functions being in membrane proliferation and wall-formation (Zachariah and Fitz-James, 1967), glycogen synthesis (Hashimoto and Yoshida, 1966) etc.

In a few fungi, some other components like cytosomes and cytoplasmic tubules have been observed, but further study is needed to know their functional significance.

### Cell Inclusions

Two main storage-products of fungal cells are (i) glycogen a polysaccharide and (ii) lipids. Of these two, glycogen is the primary storage-reserve of fungi, which incidently is the characteristic of animal cells also. It may either occur as isodiametric *beta*-particles (150-300 Å) or as rosette-like *alpha*-particles, both in vegetative and reproductive parts. Lipids, on the other hand, are the main food-reserve in spores and other reproductive bodies, and serve as the potential source of energy and carbon moieties for the synthesis of membrane etc. Glycogen and lipids both are more abundant in ageing and mature parts of the thallus. The sugar alcohol mannitol is also a common reserve carbohydrate in some fungi, specially among Ascomycetes and Basidiomycetes.

### Cell Wall and Septa

The fungal cell-wall is more akin to that of the higher plants in being a rigid structure. Most of the fungi have their cell-wall made up of Chitin, although some may have both cellulose and chitin in their wall. Cochrane (1958) has listed the wall-composition of a number of fungi. Usually the wall consists of cellulose or chitin microfibrils which are distributed in an amorphous matrix. In yeast cell-wall, however, chitin may be present only in traces and the main constituents are two polysaccharides, viz., glucan (a polymer of glucose and mannan (a polymer of mannose), which constitute about 2/3rd of the wall substances. Apart from these, various other complex substances like chitosan, amino-sugars like glucosamine, callose (a glucose polymer), heteropolysaccharides, proteins, lignin, lipids and various inorganic materials have been recorded in fungal cell-wall (Aronsan, 1965). Moreover, some enzymes may also occur in the cell-wall.

In septal morphology also, fungi show wide variations, ranging from a simple complete septum to that provided with perforations

and finally to one with elaborate ancillary structures. Complete plate-like septa are found in Phycomycetes, Hemiascomycetes and a few Deuteromycetes. Although they are thought to lack any perforations or ancillary appendages, electron microscopic studies are needed to confirm their micromorphology. In fact recent studies of a few fungi, which were earlier considered to have complete plate-like septa, have shown the presence of plasmodesmata or micropores (100-700 Å dia.), numbering as many as 50 per septum (Hashimoto *et al.* 1964; Hawker *et al.* 1966). Micropores may help in intercellular transportation. In some Mucorales, a biconvex plug has been observed (Benjamin, 1959), which is supposed to function like a valve to regulate the cell to cell protoplasmic movement.

The typical ascomycetous septum is a simple plate with a central pore (Ca 0.05-0.5  $\mu$ ), which permits free streaming of the protoplasm along with nuclear migration. In these fungi Woronin bodies like structures have been recorded, which may serve as pore-plugs.

Basidiomycetes have the most specialised and complex septa which have been designated as dolipore septa. They have a central pore which is surrounded by an annular septal swelling and is covered from both sides of the septum by thickened dome shaped membranous caps. The caps may be without pores, with numerous small pores or with large discontinuities. Moore and McAlear (1962) suggested that such a septal structure may provide humoral continuity between adjacent cells, and prohibit nuclear migration. However, nuclear migration in these fungi is now generally accepted (Snider, 1963; Raper, 1966). Therefore, it is presumed that nuclei either migrate due to their inherent plasticity which helps them to squeeze through a smaller pore, or the pore-apparatus becomes degenerated to form a simple septa allowing protoplasmic streaming.

### **MORPHOLOGICAL AND PHYSIOLOGICAL FEATURES OF FUNGI**

In morphological features also, fungi exhibit marked diversity. They range in shape and size from single celled microscopic yeast to giant multicellular mushrooms and puffballs. They grow in all types of habitats ranging from aquatic to amphibious in distribution. Many of the fungi are free living saprotrophs, but others are either strictly obligate parasites of plants, animals and man, or they live in various kinds of symbiotic relationship, with other forms of life like algae (in lichen) and higher plants (mycorrhiza).

Diversity in form has perhaps led to diversity in functions also, and

in fact, fungi appear to be physiologically well adapted to environment, nutrition etc. They can tolerate wider range of pH (pH 2.0 to 9.0), osmotic pressure, desiccation etc., as compared to bacteria. Their temperature range is also sufficiently wide. Some fungi can even grow at 0°C and cause food spoilage under cold storage, while others can thrive at 62°C and are designated as thermophillic.

Fungi possess the ability to utilize a wide spectrum of nutrients as sources of energy, carbon, nitrogen, minerals etc. Being nutritional heterotrophs, they obtain their carbon-requirements from organic carbon sources, including a variety of carbohydrate and non-carbohydrate compounds. With an effective battery of enzymes, they are able to utilize a wide array of such substances with high degree of efficiency. Some are even able to carry on heterotrophic carbon dioxide-fixation, which may, to some extent, help to replenish the cell-carbon. In nitrogen nutrition, they appear to be more versatile. They can derive their nitrogen nutrition from inorganic, ammonium as well organic sources. Even, utilization of gaseous nitrogen has been claimed for some fungi, although this needs further confirmation. Similarly, they obtain their mineral requirements from their surroundings without much of difficulty. Regarding vitamins, most of them are prolific producers of vitamins and they do not require vitamins from external sources. But a few fungal species are known to be auxoheterotrophic as they require one or the other vitamin from some external source.

Blending of all these characteristics has made fungi a heterogeneous but interesting group of organisms. Some recent investigations on their nutritional efficiency have yielded still more interesting results. Mirocha and Devay (1971) could maintain species of *Cephalosporium* and *Fusarium* on a medium devoid of any known complex organic carbon-source. These fungi assimilated carbon from labelled  $^{14}\text{CO}_2$ , when grown as heterotrophs, they were able to revert back to autotrophism, on subsequent transfer to autotrophic conditions. Autotrophism in fungi has been discussed more recently by Robinson (1973). Detailed studies on these aspects, in the years to come, may furnish still more exciting informations so that we may be able to know and understand fungi and their physiology in a better way.

## CHAPTER II

# CULTURE MEDIA

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All the living organisms require energy for maintaining their life activities. On the basis of the sources from which this energy is directly derived by an organism, two distinct groups of living beings have been recognised. Chlorophyllous plants are categorised as *phototrophs* because they possess the mechanism to trap the radiant energy of the sun directly, which is stored as chemical potential energy in the assimilated organic compounds. All the non-chlorophyllous plants as well as the animals depend upon this chemical energy. Such organisms which lack the capacity to use solar energy directly, are designated as the *chemotrophs*. Both these groups of organisms have, however, certain basic requirements of raw materials for their nutrition. Carbon, nitrogen, sulphur, phosphorus, sodium, potassium, calcium, magnesium, iron, manganese, zinc, copper, cobalt, molybdenum and vitamins are some of the important substances. Among these, carbon occupies a unique position, because compounds with carbon to carbon linkage are the characteristic features of the animate world as a whole. Green plants are able to utilize inorganic carbon in the form of carbon dioxide, which is converted to carbohydrates, through photosynthesis. The photosynthetic organisms thus, do not depend upon any other form of life, but are self reliant, as far as the carbon assimilation is concerned, and hence are designated as the *autotrophs*. The non-chlorophyllous organisms, including fungi, on the contrary, depend entirely upon the autotrophs for meeting their carbon-requirement and are therefore, categorised as the *heterotrophs*. This is because the heterotrophs are unable to harness the inorganic sources of carbon.

Nitrogen, a major constituent of proteins, nucleic acids and other cell-contents, is the next most needed element. For its utilization, it is the heterotrophs which seem to be better equipped. Fungi and bacteria exhibit considerable versatility with regard to utilization of nitrogen sources. Besides their capacity to obtain nitrogen from



organic, inorganic, or ammonium compounds, some of these micro-organisms are capable of utilizing atmospheric nitrogen also.

Sulphur and phosphorus are also fairly well distributed in all the living beings. While sulphur forms an essential constituent of some amino acids, vitamins and hormones, phosphorus occurs as a component of phospholipids, nucleic acids, etc. Phosphorus plays a significant role in phosphorylation reactions; and through the energy-rich phosphate bonds, this element helps in the transference of energy in every living system. Utilization of these two elements also differs. While phosphorus is principally obtained in the form of phosphate, the utilization of sulphur varies in different organisms. Higher plants utilize inorganic sulphur only, while fungi and bacteria are able to use both the inorganic as well as organic sources of this element. Some bacteria, possess the capacity to utilize the elemental sulphur as well.

All living organisms require vitamins and other growth factors though in minute quantity. Green plants and some bacteria are able to synthesize most of the vitamins required by them. So are many of the fungi also but a few of them exhibit their inability to synthesize some of these compounds and require preformed vitamins or their precursors in their culture media.

The mineral requirements of all the organisms are generally met by the soil, which contains these elements as inorganic salts. However, these compounds must be available in the form of solution, before they can enter into a living system. This is accomplished through the agency of water, which as a solvent not only helps in the absorption and translocation of nutrients in general, but is also essential for all metabolic processes of every living entity of this biosphere.

While formulating a culture medium for cultivation of any group of organisms, under laboratory conditions, these general nutritional requirements have to be kept in view.

## CULTURE MEDIA

First attempt to obtain laboratory cultures of fungi was made by the great Italian botanist, Micheli (1679-1737). He could succeed in growing three different molds, viz., *Mucor*, *Aspergillus* and *Botrytis*, on freshly cut surfaces of melon, quince and pear. Bulliard (1791) followed Micheli's lead and obtained cultures of *Mucor* on a paste prepared from moistened breads. Use of such substrates for artificial culture of fungi continued till Pasteur (1860) during his studies on

alcoholic fermentation, used what might be considered as an approximation of a chemically defined medium. However, it was Raulin (1869) one of Pasteur's disciples, who devised the first synthetic medium for fungi, during nutritional studies of the common mold, *Aspergillus niger*. His medium, however, was highly acidic in reaction ( $pH=2.4$ ) and had the following rather complex composition:

#### RAULIN'S MEDIUM

Water	1,500 ml
Sucrose	70.0 g
Ammonium nitrate	4.0 g
Tartaric acid	4.0 g
Ammonium phosphate	0.6 g
Potassium carbonate	0.6 g
Magnesium carbonate	0.4 g
Ammonium sulphate	0.25 g
Zinc sulphate	0.07 g
Ferrous sulphate	0.07 g
Potassium silicate	0.07 g

Since then, a large number and different kinds of media have been devised by various workers. Majority of the workers have preferred natural media, which besides being cheap are easier to prepare and are able to support a wide variety of fungi. However, such a universal medium may possibly never be devised, which may prove ideal for all the known fungal species, particularly the fastidious ones. Considerable success has, however, been achieved in the recent past in culturing some of the obligate parasites on artificial media.

Extensive work on fungal nutrition has led to the development of a large number of media, and the list to-date is long enough to merit their proper nomenclature and classification. Moreover, with increasing emphasis on biochemical and genetical aspects of fungi, the list of culture media continues to proliferate.

#### NOMENCLATURE

It has been a customary to designate culture-media after names of their formulators, chiefly to commemorate their contribution. System of naming culture media after personalities may be of some historical value but proliferation of culture media in such manner has added to confusion. Lilly and Barnett (1951) have also expressed their opinion against personality oriented nomenclature of media because in many cases they do not reflect their composition. In an attempt to develop



an alternative system of nomenclature, they have suggested the use of descriptive names based upon the carbon and nitrogen sources of the respective media. Accordingly, they prefer Czapek's or Schopfer's media to be designated as sucrose-nitrate or glucose-asparagine media respectively. That, such descriptive names carry valuable information regarding the composition of the media, can not be disputed. However, when put to general use, this method of nomenclature also does not appear to be very sound. For instance, Waksman's, Sabourand's agar and Martin's media, all will have the same designation *i.e.*, glucose-peptone medium, under this system of nomenclature. Evidently, reference to any of these media by this name will lead to uncertainties and ambiguities, as it will be difficult to ascertain as to which of the three media is being referred to. Possibly we can over-come this difficulty if a combination of the two systems is adopted. Thus a medium may be designated both after the name of its formulator as well as on the basis of its composition. Obviously, this combined system will carry the advantages of both the systems referred to above. The composition of the medium, however, should get preference over name of the formulator, and should come first in the nomenclature. For example, Czapek's medium under this system could be designated as sucrose-nitrate Czapek's agar. Identical media with similar carbon and nitrogen ingredients can thus be easily distinguished by adding the name of the formulator.

### Classification

Some of the common criteria for classifying media are their chemical composition, physical state and their empirical use. In fact, every medium is designed for a definite use and hence its physical and chemical characteristics must conform to its application and function. According to their use, media may be categorised into the following types:

(1) *Routine laboratory media.* These are media with certain complex raw materials of plant or animal origin such as yeast-extract, malt-extract, peptone etc., and are employed for routine cultivation and maintenance of a wide variety of fungi.

(2) *Enriched media.* These media are prepared by supplementing the routine laboratory media with some specific substances to meet the nutritional requirements of more fastidious organisms and are employed for their cultivation.

(3) *Selective media.* These media facilitate the isolation of a particular group of organisms or species from a mixed inoculum.

Such media contain substances which inhibit all except the desired organisms.

(4) *Differential media*. Supplemented with certain reagents or chemicals, these media aid in differentiating between various kinds of organisms on the basis of visible differences in their growth patterns. However, such type of media are used more often in bacteriological laboratories.

(5) *Assay media*. This type of medium is specifically employed for the assay of vitamins, amino acids, antibiotics, disinfectants etc., and are of definite composition.

(6) *Biochemical media*. Such media are generally used for the differentiation of micro-organisms on the basis of their biochemical activities, and are helpful in the study of their metabolic processes.

According to chemical composition media are classified into the following types:

(1) *Natural media*. A natural medium comprises entirely complex natural products of unknown composition. The raw materials of a natural medium may be of plant or animal origin, and some of the common ingredients employed for this purpose include extracts of plant and animal tissues, e.g., fruits, vegetables, egg, milk, blood, body fluids, yeast, malt and manure extracts etc. Obviously, the chemical composition and concentration of a natural medium is not well defined. On account of their complex nature, these media are able to support a variety of organisms, and hence are quite useful for routine laboratory cultures of fungi. Brefeld (1881), who was one of the pioneers in the field of fungal culture was so much impressed by the utility of some natural media he used, that he considered atleast one of them, viz. manure extract, of universal applicability for culture of fungi. Other advantages of natural media are their low cost and easier method of preparation. However, these media have certain limitations too. Due to their complex nature, their chemical composition and concentration can not be controlled. This limits their use to routine culture of fungi only, as investigations pertaining to fungal nutrition and metabolism can hardly be carried out on such media.

(2) *Semisynthetic media*. These media are so designed that some of their constituents are of known chemical composition, while others are derived from some natural sources with unknown composition. The chemical make-up of a semisynthetic medium is, thus, only partly known. Consequently, only a limited amount of control may be exercised on the composition and concentration of a semisynthetic medium, by making necessary changes in the chemically

known faction. Semisynthetic media have also limited application in physiological studies on fungi, and can best serve as a routine medium. Potato-dextrose agar is one of such accepted and popular media. Lilly and Barnett (1951) consider all agar-solidified media as semisynthetic ones, because their exact chemical make-up is partly obscured by the addition of agar-agar.

(3) *Synthetic media*. These are chemically defined media of known composition and concentration, and are exclusively composed of pure chemical substances. However, absolute purity of the ingredients is seldom achieved, although substances of only analytical reagent quality are used for such purposes. On account of their known composition as well as being in solution, these media are quite useful for nutritional and metabolic studies of fungi. The composition of these media may be amended as per requirement and as such they may be simple or complex in make-up. A simple synthetic medium contains a single carbon and energy source, a nitrogen source, generally as ammonium salt, some sulphur and phosphorus sources and various minerals. All these ingredients are dissolved in a buffered aqueous base. However, for more fastidious organisms, a complex synthetic medium is designed by incorporating some additional factors such as certain vitamins, amino-acids, purines, pyrimidines etc., or by employing a multitude of carbon and nitrogen sources together.

Media may also be differentiated according to their physical states, and may be of the following types:

(1) *Solid media*. Media in solid state are in use since the beginning of studies on fungi in the laboratory. The first laboratory culture of a fungus was obtained on a solid medium, viz. fruit slices. Some common examples of such media are nutrient impregnated slices of potato, carrot, sugar-beet etc. and coagulated egg or serum. However, with the advent of agar-agar as a solidifying agent, such media have largely been replaced by agar media. Use of fruits and vegetable slices in the cultivation of fungi is now more or less restricted to the baiting technique employed for isolation of some specific organisms.

(2) *Solid-reversible to liquid media*. Such reversible media were first introduced by Koch (1881) who observed that addition of 2 to 5 per cent of gelatin to the commonly employed media rendered them a semi-solid consistency. However, gelatin could not find a wide application on account of its low melting point (Ca 37°C), and also because it is hydrolized by many proteolytic bacteria at ordinary temperature. The use of agar-agar for solidifying culture media was

also initiated the same year and in the same laboratory. It was originally suggested by Frau Hesse (Hitchens and Leikind, 1939), whose husband worked in Koch's laboratory, and proved to be a worthy alternative to gelatin. Agar does not add to the nutritive value of a medium in any appreciable amount. It gives a transparent solid consistency to the medium at all the incubation temperatures. Besides, agar solutions of normally used concentrations melt at Ca 95°C and solidify at Ca 42°C. This facilitates the incorporation of heat-sensitive substances to such media without inactivating them. This is achieved because such heat-labile substances may be added to the medium in molten state at temperatures as low as 45°C. Apart from this, agar solidified media afford various other advantages, and are indispensable for the isolation of pure cultures as well as their microscopic examination and identification. Moreover, cultures grown on agar media afford so much convenience and ease in their handling, that these media are now widely employed for most of the routine studies on fungi.

(3) *Semi-solid media*. These are media with gelatinous consistency and are employed for specific purposes. They contain a small amount of agar or some other solidifying agent like corn-meal. These media are sometimes used for the study of motile reproductive structures of fungi.

(4) *Liquid media*. These are media without any solidifying agent, and are indispensable for most of the quantitative studies. Nutritional and metabolic studies on fungi, as well as microbiological assays are invariably carried on in liquid media. Some of the advantages of liquid media are that they permit the cultures to be aerated, the mycelium to be weighed and the metabolic products to be analysed easily. However, with respect to routine studies, liquid media have some distinct disadvantages. Growth in liquid media does not manifest the morphological characteristics of an organism. They are also difficult to handle without disturbing the culture. Moreover, liquid media are least helpful in the purification of organisms from a mixed culture. For an even distribution of nutrients and for providing uniform aeration to growing fungus, the liquid cultures are sometimes put to constant mechanical shaking.

## PREPARATION OF CULTURE MEDIA

Preparation of a medium is quite an elaborate procedure and involves several steps. Some of the important steps are detailed below:

- (A) Dissolution of prescribed amount of ingredients in an appropriate volume of distilled water. In case of a natural medium, this step involves the preparation of a decoction or infusion of the requisite material.
- (B) Adjustment of the culture solution to a suitable pH.
- (C) Dispensation or distribution of medium into suitable containers.
- (D) Sterilization of the medium by an appropriate method.
- (A) Some of the commonest ingredients of culture media and their sources are described here:

(i) *Agar*. The main constituent of agar-agar is a long chain polysaccharide, composed of *d*-galactopyranose units. However, many other components, including inorganic salts, protein-like substances etc. are also present in the form of impurity. Sometimes, long chain fatty-acids may be present in traces which may inhibit growth. Calcium and magnesium are the chief minerals associated with agar, and it is supposed that this complex substance occurs as the calcium or magnesium esters of the polysaccharide. Agar is obtained from various sea-weeds, including genera like *Gelidium*, *Euclidean*, *Pterocladia* and others. These weeds are dried, extracted by hot-water, processed, cleaned, dried and finally are made available for use either in the form of strands or powder. Agar produced at different places show some variations in their properties, e.g., the Japanese agar forms a gel of suitable firmness at 2% concentration, while its Newzealand variety does the same at 1.2% level only.

For preparation of agar media the requisite amount of agar is added and the medium is placed in a steam bath at 100°C for one hour for dissolving this compound. However, if agar is heated at low pH, it is hydrolysed to such components, which do not solidify on cooling. Media with pH of about 5.0, as are generally used for fungi, should be subjected to minimum of heating, after the agar is in an acid solution.

(ii) *Peptone*. This water soluble ingredient is obtained from lean meat or other protein materials such as heart muscle, casein, fibrin or soyabean flour, after digesting them with proteolytic enzymes like pepsin, trypsin or papain. Peptone thus prepared is available in granular powder form and its chief constituents, are peptones, proteoses, amino acids, various inorganic salts, like phosphates, magnesium and potassium, and some accessory growth substances including riboflavin and nicotinic acid. Peptone powder is highly hygroscopic and becomes sticky on exposure to air. Different grades

of peptone are available for specific purposes, *e.g.*, bacteriological peptone, mycological peptone, proteose peptone, neopeptone etc.

(iii) *Yeast extract*. It is available in the form of a dark sticky paste with approximately 70% solid contents. It contains a wide variety of amino-acids, which comprise nearly half of its mass; growth substances, particularly of the Vitamin B group; inorganic salts like potassium, phosphate, and some carbohydrates, like glycogen, trehalose and pentoses are also present in this ingredient which comprise more than 10% of its constituent.

Yeast-extract is obtained from baker's and brewer's yeast. The cells are washed and allowed to autolyze by mild heating, *e.g.* at 55°C. Some times, the cells are hydrolysed with hydrochloric acid or a proteolytic enzyme. Subsequently, the cell-walls are removed by centrifugation or filtration and the extract thus obtained is concentrated to a thick dark paste, ready for use.

(iv) *Meat-extract*. This has proved a convenient substitute for the infusion of fresh meat used before. Commercially known as Lab-Lemco, meat extract is prepared by boiling finely divided lean beef in water, so that water soluble ingredients pass into solution. The solution is concentrated to a dark sticky paste, after removing the excess of fat. The paste so prepared contains about 70-80 per cent of solids, including protein degradation products like peptones, proteoses, amino acids and gelatin, besides other introgenous compounds such a purines, creatine, creatinine, carnosine, anserine etc. mineral salts like  $\text{KH}_2\text{PO}_4$  and  $\text{NaCl}$ , growth factors, *e.g.*, thiamine, nicotinic, riboflavin, pyridoxin, pantothenic acid etc., and some carbohydrates.

(v) *Case in hydrolysate*. It is a hydrolytic product of the milk-protein casein, and contains amino acids, phosphate, mineral salts and certain growth factors. It is a good substitute for peptone, because its composition is much more defined than that of peptone, and thus may be of particular use where a nearly defined medium is required. Casein hydrolysate prepared through enzymatic (trypsin) hydrolysis is more useful, as it contains full range of amino acids and abundant tryptophane. On the contrary, the one prepared by the action of hydrochloric acid is nutritionally poorer as tryptophane is completely destroyed during hydrolysis and some other amino acids are also quantitatively reduced.

## (B) Adjustment of pH

Fungi, like other micro-organisms are very much susceptible to



the reaction of the surrounding medium upon which depend their growth and survival. Some of the fungi are very much specific with regard to the reaction of the medium while others tolerate a wider range of acidity or alkalinity. However, all have some particular reaction, at which they attain their optimal growth. Fungi exhibit differences over bacteria and actinomycetes in that they are comparatively better adapted to invade acid environment but this does not mean that fungi are restricted to such environments only.

The reaction of any liquid is expressed in terms of hydrogen ions present in it, since that is easier to be measured; similarly for the reason of practical convenience hydrogen ions are expressed on logarithmic scale or pH scale. Thus, pH value of a liquid is the logarithm of the reciprocal of the hydrogen ion concentration, *i.e.*,

$$\text{pH} = \log \frac{1}{\text{H}^+}$$

$$\text{For neutral water, } \text{pH} = \log \frac{1}{10^{-7}} = 7.0$$

( $\text{H}^+$  of pure water at  $22^\circ\text{C}$  is  $1 \times 10^{-7}$  gram ions per litre). However, for a clear understanding of the pH scale, the following two points must be emphasised:

(i) Since it is a logarithmic scale, a change in one unit of pH will actually represent a tenfold shift in the hydrogen ion concentration. For example, a liquid at pH 4.0 is ten times more acidic than at pH 5.0 or it is ten times more alkaline at pH 10.0 than what it will be at pH 9.0.

(ii) On account of its reciprocal nature, the lower the pH value, the higher will be the acidity. Actually, pH values below 7.0 indicate acidity while beyond 7.0 indicate alkalinity.

For measurement and adjustment of pH of culture-media several methods are employed, *viz.*, (a) electrometric methods, (b) indicator dye method, (c) colorimetric method. Among these techniques, the electrometric method is the most accurate in which potentiometers are employed for pH determination. However, use of a wide range of indicator dyes and indicator impregnated pH-papers are now preferred for routine purposes. The colorimetric method, although employed quite frequently due to its simplicity, is the least accurate among these three techniques.

While adjusting the pH of the culture media at the optimum value for the organism concerned, it has to be kept in view that micro-organisms attack the medial constituents and thus are able to cause a shift in the acidity or alkalinity of the medium, sufficient to even kill them. To avoid such a situation, or to minimize pH changes in the

medium, certain buffers are incorporated in the medium. These buffers are compounds which have the capacity to resist pH changes. Two such compounds, which are widely employed as buffers, are  $K_2HPO_4$  and  $KH_2PO_4$ . Some other ingredients of the media like peptone also possess some buffering capacity.

### (C) Dispensation of the Medium

The next step in the preparation of culture media is their distribution into suitable containers, like Erlenmeyer flasks stoppered with cotton-wool, test tubes with cotton-plugs or metal caps, screw-capped bottles, of different shapes and sizes and Petri-plates. Routine media not containing any heat-labile substances are dispensed into containers and are subsequently sterilised. However precautions are necessary, if an ingredient of the medium to be distributed is heat-labile. In such cases, the heat-stable ingredients are prepared and sterilised as usual, the unstable components separately and suitably sterilised, both the fractions are then mixed and dispensed subsequently under sterile precautions, into sterile containers. A suitable apparatus for distributing media under sterile conditions is shown in Fig. 2.1. The whole apparatus is wrapped in paper and sterilised. The Petri-plate lid over the funnel protects the sterile medium in the funnel and the hood attached to the tube protects the medium as it is distributed to the containers. In an alternative method, the heat-stable ingredients are distributed and sterilised, to which is added the sterile unstable component from a sterilised graduated pipette or an apparatus specifically designed for that.

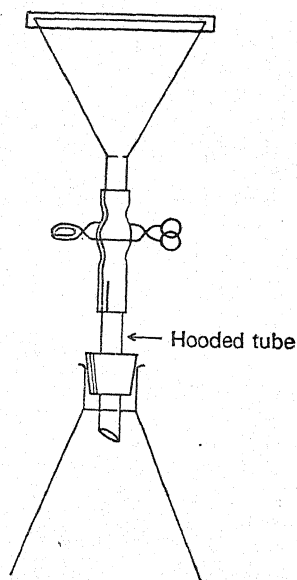


Fig. 2.1. Apparatus for distribution of media with sterile precaution.

### (D) Sterilisation of Media

Culture media as well as the glass wares may be sterilised in a variety of way, which fall under the following categories:

#### I. Physical Methods:

##### 1. Heat:

- (i) Dry heat (including infra-red radiation),



- (ii) Moist heat.
  - 2. Radiation:
    - (i) Ultra-violet radiation,
    - (ii) Ionising radiation.
  - 3. Filtration.
- II. Chemical Methods.

### (I) STERILISATION BY PHYSICAL METHODS

#### 1. Sterilisation by Heat

Heat may be utilised for sterilisation either in dry or moist form. However, moist heat is much more effective and requires both shorter duration and lower temperature. Sterilisation by moist heat generally is complete at  $121^{\circ}\text{C}$  for 15-30 minutes exposure. On the contrary, sterilisation by dry heat requires a temperature of  $160^{\circ}\text{C}$  for 60 minutes. The two kinds of heat treatments kill the micro-organisms possibly by coagulating and denaturing their enzymes and other proteins. Therefore culture media must be sterilised by moist heat, because they contain water. The dry heat, on the contrary, probably causes a destructive oxidation of the cell constituents and thus kills the organisms. Sterilisation by dry heat is employed for rendering sterile glass-wares, metal instruments etc. as also for sterilising anhydrous fats, oils, etc. On account of their impermeability to moisture.

(i) *Application of dry heat.* Sterilisation by dry heat may be achieved by different methods according to the requirements:

(a) *Red heat.* Heating to redness is employed for sterilising inoculating needles, forceps, spatula etc.

(b) *Flaming.* Scalpels, needles, mouths of culture vessels, cotton-plugs, glass-slides and cover-glasses are sterilised by this method, by passing them through the flame. Sometimes, these articles are immersed into methylated spirit and the spirit is burnt off, but such treatment does not produce a temperature sufficient to affect complete sterilisation.

(c) *Hot-air-oven.* Heating in an air-oven is employed for sterilising all types of glass wares, instruments like forceps, scissors etc., and materials that are impermeable to moisture, like powders, fats, oils and greases. Generally one hour exposure to a temperature of  $160^{\circ}\text{C}$  is considered sufficient, but a  $2-2\frac{1}{2}$  hours exposure is sometimes essential, especially when slowly heating materials, such as powders, oils etc. are sterilised or when the air-oven is over-loaded and is without a circulating fan.

(d) *Infra-red-radiation.* Recently, infra-red radiation has been

employed for dry heat-sterilisation. With the help of an infra-red electric lamp, the object to be sterilised is subjected to a temperature of about  $180^{\circ}\text{C}$ . In special chambers, temperatures above  $200^{\circ}\text{C}$  may be attained *in vacuo* by this method for sterilising certain instruments.

(ii) *Application of moist heat.* Moist heat may be applied at different levels *viz.* (a) at temperatures under  $100^{\circ}\text{C}$ , (b) at  $100^{\circ}\text{C}$  and (c) at temperatures above  $100^{\circ}\text{C}$ . However, as indicated earlier, complete sterilisation is affected only by the third procedure, which is generally used for the sterilisation of culture media. Heating at and below  $100^{\circ}\text{C}$  have limited application like in pasteurisation of milk, sterilisation of serum and vaccines, boiling of rubbertubes, stoppers, forceps, scissors, etc. However, Tyndallisation or intermittent exposure at  $100^{\circ}\text{C}$  for three successive days have found its utility in the sterilisation of some types of culture media *e.g.* those containing sugars, which may get decomposed at higher temperature, and for gelatin media, which may fail to solidify on cooling if subjected to higher temperatures. However, it must be borne in mind that many thermophilic, anaerobic bacteria may not be killed by this procedure, because the medium may not be suitable as well as the conditions may not be favourable for their spores to germinate.

Moist heat above  $100^{\circ}\text{C}$  is generally applied through the use of the autoclave. Autoclaving is now the most dependable and commonly employed method of sterilisation of culture media and is based on the following principle. When the vapour pressure of water equals the pressure of the surrounding atmosphere, the water boils. Under normal atmospheric pressure this takes place at  $100^{\circ}\text{C}$ . However, if water is boiled in a closed vessel the inside pressure increases, which also causes a rise in its boiling point as well as the temperature of the steam it forms. Thus the autoclave provides a means for subjecting the culture media and other articles to moist heat at temperatures above  $100^{\circ}\text{C}$ . Generally the media are sterilised by autoclaving them at a temperature of  $121^{\circ}\text{C}$ , which is achieved at 15 lb. per sq. inch gauge pressure, for a minimum holding time of 12 minutes. For greater dependability, a 50 per cent safety period is added to make the total duration of the holding time to 18 minutes. It need not be mentioned here that the holding period is timed as beginning when the pressure gauge first records a pressure of 15 lb. per sq. inch. Different kinds of autoclaves have been developed, of which the simple non-jacketed ones are most commonly used in the laboratories. The steam-jacketed autoclaves with automatic air and condensate discharge systems are much more advantageous, although a bit expensive. Similarly, high pre-vacuum sterilisers, which are

presently the most advanced types are equipped with exhaust pumps that are able to drive out more than 98 per cent of the air and thus create almost complete vacuum. This enables the steam to penetrate very rapidly and heat up the entire load very quickly and thoroughly.

## 2. Sterilisation by Radiation

Both ultraviolet radiations and ionising radiations are in use as sterilising agent. Ultra-violet radiations of less than  $330\ \mu$  wave-length are able to kill the micro-organisms. The germicidal action of sun-light is mainly due to the ultraviolet rays present in it. Artificially, ultra-violet rays of more effective wave-lengths, such as  $240\text{--}280\ \mu$  are produced by mercury vapour lamps. Such lamps are generally used in the laboratory for sterilising the inoculation-chambers or rooms.

Ionising radiations including, X-rays,  $\gamma$ -rays and high speed electrons are also able to kill micro-organisms in adequate doses. However, the necessary apparatus required to produce these radiations, are too expensive to be used frequently.

## 3. Sterilisation by Filtration

This technique employs special type of filters having pores so small that ordinary bacteria are arrested. This method is particularly useful for sterilising heat-sensitive materials, such as culture-media containing serum, antibiotic solutions, culture-filtrates etc. The average pore diameter of such filters is  $0.75\ \mu\text{m}$  or less. The various kinds of such filters now in use are:

- (i) Berkefeld filters made up of fossil diatomaceous earth,
- (ii) Chamberland filters, made up of unglazed porcelain,
- (iii) Seitz filters, consisting of an asbestos disk,
- (iv) Sintered glass filters, made up of finely ground glass, and
- (v) Cellulose membrane filters, made up of cellulose nitrate or acetate.

## (II) STERILISATION BY CHEMICAL METHODS

Chemical agents, like chloroform, lysol, mercuric chloride, formaldehyde, ethylene oxide etc. are also used for sterilisation but they are chiefly employed as disinfectant, and have practically no use in the sterilisation of culture media.

## COMPOSITION OF SOME COMMON LABORATORY MEDIA

### NON-SYNTHETIC MEDIA

#### *Corn Meal Agar.*

Corn meal	20	g
Peptone (if desired)	20	g
Dextrose (if desired)	20	g
Agar	15	g
Distilled water	1000	ml.

#### *Oat Meal Agar.*

Oats	100.0	g
Agar	15.0	g
Water	1000.0	ml.

#### *Potato Dextrose Agar.*

Peeled potato	250.0	g
Glucose	20.0	g
Agar	15.0	g
Water	1000.0	ml.
pH 6.0 to 6.5		

#### *Soil Extract Agar.*

*Soil extract	100.0	g
Glucose	1.0	g
Dipotassium phosphate	0.5	g
Agar	15.0	g
Tap water	900.0	ml.
pH 7.0 to 7.2		

[\*Steam 1000 g of garden soil in one litre of tap water in an autoclave at 15 lb pressure for 30 minutes. Add a small quantity of calcium carbonate and filter the soil suspension through double layer of filter-papers. The filtrate is sterilized in a flask and stocked, for future use.]

*Malt Extract Agar.* Useful for the growth of wood-destroying and many other fungi.

Malt extract	25	g
Agar	15	g
Distilled water	1000	ml.

#### *Martin's Rose Bengal Streptomycin Agar.*

Dextrose	10.0	g
Peptone	5.0	g
Potassium dihydrogen phosphate	1.0	g
Magnesium sulphate	0.5	g

Rose Bengal	
(1 part in 30,000 parts of the medium)	
Agar	20.0 g
Streptomycin	0.03 g
Distilled water	1000 ml.

*Waksman's Medium.* This is specially used for counting soil fungi.

Glucose	10 g
Peptone	5 g
Potassium hydrogen phosphate	1 g
Magnesium sulphate	0.5 g
Agar	20 g
Distilled water	1000 ml.
pH 4	

*Sabouraud's Medium.* This medium is good for many fungi including human or animal pathogens.

Glucose (or Maltose)	40 g
Peptone	10 g
Agar	15 g
Distilled water	1000 ml.

#### SYNTHETIC MEDIA

*Czapek's Solution.*

Sucrose	30.00 g
Sodium nitrate	2.00 g
Dipotassium phosphate	1.00 g
Magnesium sulphate	0.50 g
Potassium chloride	0.50 g
Ferrous sulphate	0.01 g
Distilled water	1000.00 ml.

*Coon's Medium (For Fusarium).*

Saccharose	7.20 g
Dextrose	3.60 g
Magnesium sulphate	1.23 g
Potassium acid phosphate	2.72 g
Potassium nitrate	2.02 g
Water	1000.00 ml.

Add 25 ppm solution of malachite green or 40 ppm gentian violet.

*Richard's Solution.*

Potassium nitrate	10.00 g
Potassium dihydrogen phosphate	5.00 g
Magnesium sulphate	2.50 g
Ferric chloride	0.02 g
Sucrose	50.00 g
Distilled water	1000.00 ml.
pH 6.6 to 7.2	

## CHAPTER III

# ENZYMES

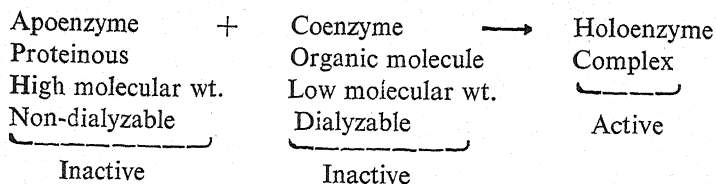
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Enzymes may be regarded as the vital constituents of cells, since any interference in their activity is reflected by some change in the cell, which may even lead to the death of the organism. Dixon (1949) sounded it in a more direct fashion, when he observed that "the life processes of organisms are controlled and directed by a complicated and interrelated series of enzymes or enzyme systems."

The concept of catalytic action of enzymes in living system had its origin in microorganisms particularly in yeast. After Pasteur (1860) established fermentation as a physiological process of microbes, it was Traube who mooted the idea that the cells contained some "ferments" which caused fermentation. Definite experimental evidence for this hypothesis was provided by Buchner (1897), which ultimately led to the concept that all reactions of the living cells are carried out under the influence of ferments formed by the living cell, but capable of acting independently of the cells. The contribution of yeasts in the formulation of the enzyme theory was fully acknowledged by Kuhne, who coined the word *enzyme* from the Greek phrase, *en zyme*, meaning in yeast.

### CHARACTERISTICS OF ENZYMES

Enzymes have been defined as complex organic catalysts of biological origin and are simple or conjugated proteins containing some other chemical groups. The entire enzyme complex is designated as holoenzyme, which comprise a proteinous component the apoenzyme and a low molecular weight organic molecule the coenzyme or the prosthetic group.



The non-protein component of enzymes may in some cases be metals (Fe, Mn, Zn, etc.), acting as inorganic coenzymes, without which some of the enzymes are inactive. These metal ions are also called as co-factors, or activators. Examples of enzymes of various constitution are tabulated below:

TABLE 3.1  
SHOWING CONSTITUTION OF SOME ENZYMES

Enzymes	Constitution		
	Apoenzyme	Coenzyme (organic)	Cofactor
Urease	+	—	—
Catalase	+	—	+
Dehydrogenases	+	+	+

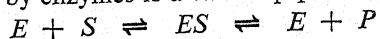
Due to their proteinaceous nature, they are unstable substances, and are easily denatured by heat, precipitated by ethanol or concentrated solution of inorganic salts like ammonium sulfate etc., and do not dialyze through semipermeable membrane.

Another striking feature of enzymes, viz. specificity is also largely due to their proteinaceous constitution. On account of their specificity they are very selective, any single enzyme would react with only a single substrate or in some cases with particular chemical groupings or chemically related substances.

As catalyst enzyme molecules are highly efficient in accelerating the conversion of substrate into its end product. A single enzyme molecule can transform as many as 10,000 to one million of substrate molecules per minute, without itself undergoing any change or alteration. It is obviously on this account that exceedingly minute quantities of enzymes are sufficient for cellular processes.

## MECHANISM OF ENZYME-ACTION

This fascinating group of compounds have attracted considerable attention of physiologists and biochemists. However, much remains to be learnt about their mechanism of action. Several theories have been proposed, all of which have atleast a common concept that the reaction catalyzed by enzymes is a two step process as shown under:



Where  $E$  stands for enzyme,  $S$  for substrate,  $P$  for product and  $ES$  for enzyme-substrate complex. Both the steps are reversible, which indicate that all the enzymes are potentially effective both in dissimilatory as well as assimilatory phases of a reaction.

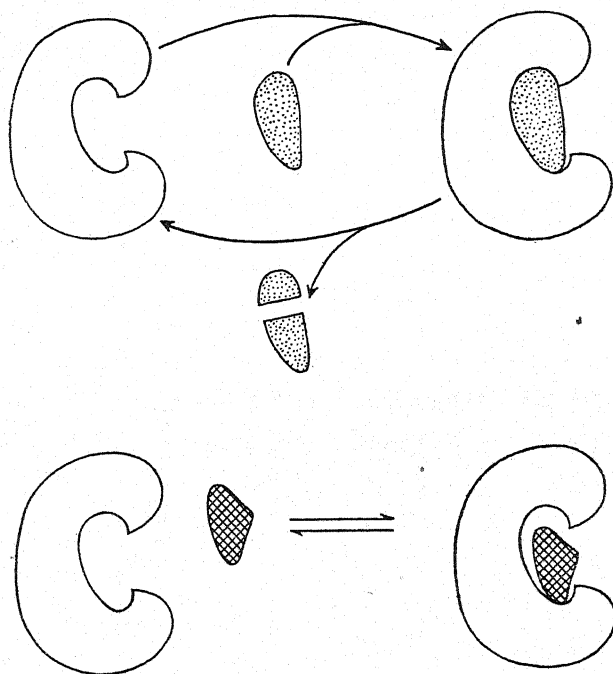


Fig. 3.1. Lock and key model of enzyme action.

Recent crystallographic studies with some purified enzymes, like chymotrypsin (Blow *et al.* 1969), lysozyme, (Phillips, 1966), carboxypeptidase (Blow and Steitz, 1970) etc. have indicated three different possibilities regarding the manner in which the enzymes can combine with their substrate.



(1) The lock and key hypothesis, according to which the substrate must have the right shape to fit into the appropriate hole, or lock on the enzyme surface. When the substrate is transformed into products, the latter dissociate from the enzyme (Fig. 3.1).

(2) According to another view the substrate exhibits high chemical (electronic) affinity for certain areas of the enzyme surface, called active centres. Binding to this area leads to small distortions in the structures of the enzyme and particularly the substrate. This strain ultimately leads to the splitting of or change in the substrate molecule. The altered molecules loose their affinity for the active centre and hence are released. The free enzyme again combines with fresh molecules of the substrate and the cycle is once again repeated (Fig. 3.2).

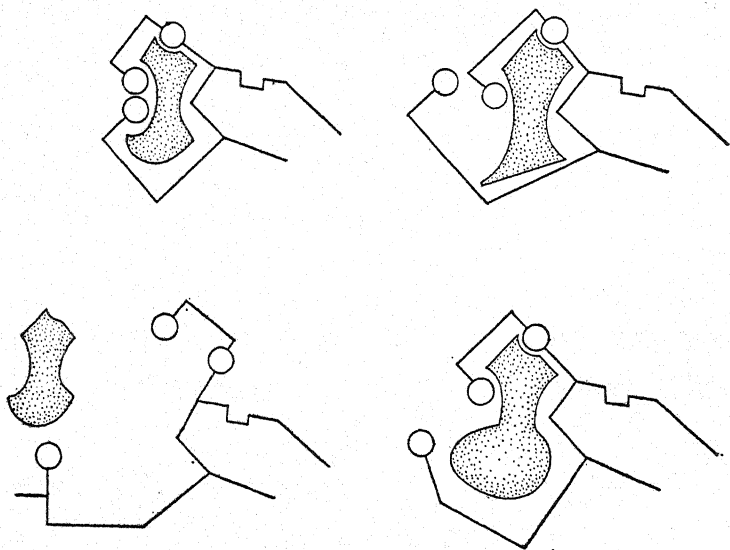


Fig. 3.2. Induced fit model of enzyme action.

(3) This theory postulates that an appropriate substrate may induce an active configuration in the enzyme by effecting a conformational change. Actually the substrate can not enter the active centre of the enzyme, unless it causes a change in the conformation of the enzyme. This type of combination has been termed as embracing mechanism in carboxypeptidase by Blow and Steitz (1970).

## NOMENCLATURE AND CLASSIFICATION OF ENZYMES

The fact that enzymes exhibit marked substrate specificity, is in itself an indication that the cells possess a great variety of enzymes to carry out its multitude of reactions and therefore they must be properly designated and classified. Two methods of nomenclature of enzymes are in practice, the one based on the name of the substrate; and the other denotes the type of reactions they catalyze. The following tabulated account illustrates both these systems of nomenclature:

### I. Nomenclature on the Basis of Substrate

#### (a) SUBSTRATE : GROUP OF COMPOUNDS

<i>Substrate</i>	<i>Enzymes</i>
Protein	Proteinase
Carbohydrate	Carbohydrase
Lipid	Lipase

#### (b) SUBSTRATE: SPECIFIC COMPOUND

<i>Substrate</i>	<i>Enzyme</i>
Cellulose	Cellulase
Starch (amylose)	Amylase
Lactose	Lactase
Sucrose	Sucrase (or invertase)

### II. Nomenclature on the Basis of Reactions

<i>Reaction</i>	<i>Enzyme</i>
Oxidation	Oxidase
Reduction	Reductase
Decarboxylation	Decarboxylase
Removal of hydrogen	Dehydrogenase

As is clear from above, while naming an enzyme it is the customary to add a suffix—*ase* either to the substrate or to the kind of reaction involved although with a few exceptions, like the enzymes papain, pepsin, etc.

Classification of enzymes has been attempted along various lines, namely on the basis of (A) nature of the enzyme, (B) site of action and (C) the type of reactions, etc.

(A) On the basis of nature of the Enzyme Karstrom (1938) recognised two broad groups of enzymes.

(1) Constitutive enzymes, and (2) Adaptive enzymes.

(1) *Constitutive enzymes*. Enzymes, which are always produced by the cells, independently of the constitution of the medium or the substrate on which the cells are growing.

(2) *Adaptive (inducible) enzymes*. Enzymes, which are produced only in the presence of a specific substrate, or some chemically related substance called inducer. The non-substrate inducers are sometimes more efficient than the normal substrate. For example, Halvorson (1960) found that methyl- $\beta$ -glucoside is about forty times more active inducer of  $\beta$ -glucosidase in yeast than is cellobiose. Some inducers can not serve as substrate of the enzyme, and are called "gratuitous inducers," e.g. thiogalactosides inducing  $\beta$ -galactosidase in yeast. However, it is now believed that the inducible enzymes also preexist in the cell, but in such a minute quantity which can not be detected by our present techniques. Prolonged incubation on appropriate substrate, therefore, leads only to their accelerated synthesis. This view has been supported by experiments showing similar enhancement in the production of constitutive enzymes also in presence of certain specific substrates as in the case of  $\beta$ -glucosidase of yeast (MacQuillan and Halvorson, 1962 a).

Induction of enzymes is a well documented and important phenomenon in a variety of fungi. They are able to produce various inducible enzymes in presence of very minute quantities of inducing substances. A few examples which have been extensively investigated include, amylase, inulase, maltase, sucrase, lipase, pectin-polygalacturonase, tannase, etc.

Informations on adaptive nutrition in fungi have largely been recorded in yeasts and *Neurospora*. Spiegelman and his associates studied the induction of galactozymase and maltase in yeast (Spiegelman and Halvorson, 1953). Spiegelman (1945) reported that *Saccharomyces carlsbergensis* under aerobic conditions adapted to galactose-utilization only in about 30 minutes, while nearly 20 hrs. were required for the same under anaerobic conditions. Respiratory enzymes, in yeast, are induced by molecular oxygen (Slonimski, 1956). When fermenting yeast suspension was aerated, enzymes like cytochrome c oxidase, cytochrome c per-oxidase as well as catalase and cytochrome c exhibited 200-fold, 50-fold, and 30-fold enhancement respectively. Similarly, induction of maltase in yeast occurred without any exogenous nitrogen, obviously from the free amino acid pool of yeast, which may require the breakdown of the preexisting enzymes. In fact, inductions of enzyme activity could occur only under normal conditions of protein-synthesis, and anaerobic or nitrogen-starved yeast cells either totally failed or exhibited very

mild inducing abilities.

Induction of enzyme activity perhaps involves a genetic mechanism. Spiegelman (1950) derived this idea from the concept that genes function by controlling the enzyme synthesis. He indicated that the presence of a particular gene in the genetic make-up of an organism does not necessarily mean that the enzyme regulated by that gene will always be present in the cell. Rather, the concerned gene only ensures that the cells carrying it have the potentiality to produce that particular enzyme, and obviously the surrounding or the substrate on which the cell is growing is one of the major factors in the initiation of this enzyme activity. Thus in all cases of induction, the cell contains the genes required to determine the amino acid sequence of the concerned enzyme, and induction simply activates the genes. The inducible and constitutive  $\beta$ -glucosidase of yeast are controlled by the same structural gene and the two enzymes are identical on various enzymological criteria.

Studies on induction of galactozymase in the presence of galactose in yeasts, have shown that the enzymes galactokinase, transferase, and epimerase, which are induced simultaneously, are controlled by three closely linked genes, *ga*, *ga*<sup>7</sup>, and *ga*<sup>10</sup> (De Robichon-Szulmajster, 1958; Douglas and Hawthorne, 1964). A similar mechanism is known in the bacterium *Escherichia coli*, where groups of linked genes control the enzymes at different steps of a pathway, under an overall control of an operator gene. Such an 'operator' gene has not so far been located in yeast. According to Jacob and Monod (1961), who studied the induction and repression of  $\beta$ -galactosidase in *Escherichia coli*, the 'operator' gene initiates RNA synthesis, while a 'regulator' gene produces a repressor, which keeps the operator gene inactive. The inducer is believed to combine with the repressor substance and thus the operator gene is free to produce the RNA essential for enzyme synthesis. It is presumed that an unstable messenger RNA is produced, which interact with ribosomes in enzyme synthesis.

(B) On the basis of site of action two groups of enzymes are recognised, viz. (i) intracellular or endoenzymes, functioning within the cell and (ii) extracellular or exoenzymes, functioning outside the cell. However, it must be emphasized that all enzymes are initially produced inside the cell, and the extracellular enzymes are those which are excreted through the cell-wall to function in the immediate surroundings of the cell. Thus the two groups perform somewhat different functions. While the extracellular enzymes bring about necessary changes in the nutrients in the vicinity of the cells, and render them diffusible into the cell, the intracellular enzymes are concerned

with the assimilation of cellular substances as well as with energy providing catabolic reactions of the cell.

Extracellular enzyme production by fungi is well known, and in fact a large number and kinds of such enzymes have been recorded in various fungal forms. Davies (1963) has summarised the extracellular enzymes of many fungi, while Reese (1963) has recorded the extracellular cellulolytic and related enzymes of fungi. To mention only one example, the fungus *Aspergillus niger* is able to produce a multitude of carbohydrases, including  $\alpha$ -amylase  $\alpha$ -glucosidase, glucoamylase, limit dextrinase, cellulases,  $\beta$ -glucosidases,  $\beta$ -1 : 6 glucan hydrolase, endopolygalacturonase, exopolygalacturonase, invertase,  $\beta$ -1 : 4 xylanase and pentosanase, besides various other enzymes to degrade other groups of compounds.

Enzymes are macromolecular compounds and therefore two pertinent questions regarding the extracellular enzymes have received due attention: (i) How these large molecules are released from inside the cell, once they are synthesized, and (ii) What is the site of release for these compounds.

Regarding the mechanism of release of extracellular enzymes, it is not yet clear whether such enzymes are produced inside the fungal cell and transported out through the cell membrane, or they are synthesized at the surface of the cell and subsequently released into the surroundings. Contradictory reports are available in this regard. In *Polystictus versicolor*, Lindeberg and Holm (1952) reported that the polyphenol oxidase enzyme within the hyphae resembled its extracellular counterpart. A similar situation was recorded in *Fomes annosus* (Nokrans, 1957). Such observations indicated an intracellular synthesis of the exoenzymes followed by a release through the membrane. However, contrary evidences have also been recorded, among which the enzyme invertase\* is probably the most thoroughly investigated. Invertase produced by yeast and *Neurospora* conidia was found principally associated with the membrane (Burger *et al.*, 1958, 1961; Metzenberg, 1964). Similarly, invertase of some other fungi, like *Myrothecium verrucaria*, ectotrophic mycorrhiza of beech and fungal component of the lichen *Peltigera polydactyla* (Mandels, 1953; Harley and Smith, 1956; Harley and Jennings, 1958), also appear to be located at the surface. Same is the case with invertase produced in the spores of many other fungi (Mandels, 1953; Metzenberg, 1963). Trevithick and Metzenberg (1964) observed that invertase activity was lost, or much of the yeast invertase was released as soluble enzyme

\*Also referred as sucrase and saccharase.

(Islam and Lampen, 1962) as soon as the walls were disintegrated, although the intact protoplasts could then produce and liberate the enzyme. The enzyme in such protoplasts exhibited the same characteristics as that obtained extracellularly (Lampen, 1965; Trevithick and Metzenberg, 1964). Further, association of mannan, glucosamine and hexosamine with the enzyme is yet another evidence supporting a membrane bound location of this enzyme, because of the occurrence of these substances as normal components of yeast cell-wall (Northcote and Horn, 1952; Lampen, 1965).

There are some suggestions that the extracellular enzymes may exist in a region physiologically outside the permeability barrier. However, the concept regarding the location of this region which is often referred to as 'free space' is not clear. Briggs *et al.*, (1961) defined this 'free space' as "the phase in the cell or tissue into which solutes move relatively freely, and was distinguished from the osmotic volume into which solutes, but not the solvent penetrate relatively slowly". Conway and Downey (1950) tried to estimate the free space of yeast cells, but no data on this line are available for filamentous fungi, and, therefore, no definite inferences could be drawn.

The second question, regarding the site of liberation is still more in doubt. Release of enzymes like celluloses with molecular weights of 55,000, 30,000 and 5,300 (Selby and Maitland 1965) poses the real problem. Very little evidences are at hand in this regard. In some similar cases, like release of anthraquinone fragments in *Fusarium* spp., indigo by *Schizophyllum commune* etc., release by the autolysis of older parts of the mycelium was indicated. However, this definitely cannot be a general mechanism. Evidences for secretion by the hyphal tips are also scanty. Similarly, release of invertase (mol. wt. Ca 30,000) by pinocytosis has been doubted by Holter (1965).

Thus, much more investigations regarding the actual site of synthesis as well as their release is needed, before any definite hypothesis could be formulated.

### **Locations of Some Fungal Enzymes**

Locations of some of the enzymes in various-parts of fungal cells as reported by various workers are given in the following list:

(a) *Pyruvate oxidase:*

In mitochondria; the soluble fraction in the supernatant oxidizes pyruvate *via* acetaldehyde and acetate (Holzer and Goedde, 1957) and not to acetyl CoA.

- (b) *Succinic dehydrogenase*: Exclusively in mitochondria.
- (c) *Cytochrome oxidase*: In mitochondria.
- (d) *Peroxidase*: In mitochondria (Zalokar, 1960).
- (e) *Polyphenol oxidase*: In sporangial cell-walls (Cantino and Horenshtein, 1956).
- (f) *L-glycerophosphatase*: Between mitochondria and supernatant (Maruyama and Alexander, 1962).
- (g) *Glucose 6-phosphatase*: Chiefly in the supernatant (Maruyama and Alexander, 1962 a).
- (h) *Disulphide reductase*: In mitochondria of yeast (Nickerson and Falcone, 1956 a, b).
- (i) *NAD (P) specific cytochrome reductase*: Mainly mitochondrial but in *Fusarium* in supernatant also (Maruyama and Alexander, 1962 a).
- (j) *Alkaline phosphatase*: In supernatant cytoplasm (Zalokar, 1960).
- (k) *Acid phosphatase*: In cytoplasm, mitochondria cell membrane or cell-wall. In centrifuged cells mainly distributed in mitochondria (Zalokar, 1960). De Duve *et al.* (1962) suggest that lysosomes contain all acid phosphatase. In yeast, this enzyme is mostly located near the cell membrane (Schmidt *et al.*, 1963).
- (l)  *$\beta$ -Galactosidase*: In mitochondria (Zalokar, 1960) but easily extractable in enzyme preparations (Landman Bonner, 1952).
- (m) *Invertase, cellobiase, trehalase and maltase*: All located at the surface of fungal spores (Mandels, 1953).
- (n) *Invertase, melibiase*: Yeast cell surface (Fries and Ottolenghi, 1959 a, b).
- (o) *ATPase*: In mitochondria, but more than 80% bound to endoplasmic reticulum (Bolton and Eddy, 1962) and therefore in supernatant (Iwasa *et al.*, 1959).
- (p) *Fumerase and aconitase*: In yeast particles (mitochondria).
- (q) *Aldolase, aconitase, and uricase*: Mainly in supernatant in *Fusarium* (Maruyama and Alexander, 1962 b).

(C) The type of reactions catalyzed would obviously be the best criterion for classifying enzymes, since one enzyme is distinguished from the other on this very specific characteristic. Fortunately, this fact was realised and in 1961 the international union of Biochemistry adopted a comprehensive scheme of classification and nomenclature prepared by the International Commission on Enzymes set up specially for the purpose in 1956.

On the basis of the chemical reactions catalyzed by the enzymes, the commission identified six groups of enzymes, which have been shown in Table 3.2.



TABLE 3.2

## MAJOR GROUPS OF ENZYMES AND THEIR ACTIVITIES

<i>Enzyme-groups</i>	<i>Types of reaction</i>
Oxidoreductase	Removal or addition of electrons with resulting loss or gain of protons.
Transferases	Transfer of a group from one molecule to another.
Hydrolases	Addition of water leading to the splitting of a bond.
Lyases	Removal of a group to cause formation of a double bond, or addition of a group to a double bond.
Isomerases	Rearrangement of a molecule into different structural isomer.
Ligases of Synthetases	Synthesis of a new molecule from two precursors.

Based on this classification, the commission has also finalized a scheme for numbering of enzymes. Under this system, each enzyme is assigned a four figure number separated by points. The four digits denote respectively the class, the sub-class, the sub-sub-class and the serial number of the enzyme in its sub-sub-class. The main advantage of such a system of numbering is that it leaves ample scope for incorporation of new enzymes as and when they are discovered, without disturbing those already recorded.

A list of a enzymes including those recorded in fungi alongwith their systematic and trivial names, enzyme commission number and some related references has been tabulated by Dixon and Webb (1967).

**FACTORS AFFECTING ENZYME ACTIVITY**

A variety of physical and chemical conditions are known to influence the activity of enzymes, which may be discussed under the following heads:

1. Temperature.
2. Hydrogen-ion-concentration of pH.
3. Enzyme/Substrate/Product ratio.
4. Chemical agents.

### (1) Temperature

Enzymes show varied response to temperature, depending upon the range of temperature as well as the enzyme concerned. Like other chemical reactions, the enzyme catalyzed reactions also increase with temperature. However, the temperature coefficient ( $Q_{10}$ ) of most of the enzymatic reactions is less than two, and the stimulatory effect is limited to a small temperature range. Enzymes show different degrees of sensitivity to heat and each enzyme has an optimal temperature of its own for its activity. But most of the enzymes are destroyed by exposure to boiling even for a few minutes. At temperatures above optimum the enzyme is inactivated which may initially be reversible but later may become irreversible. According to Bayliss (1925), the optimum temperature is that at which the increased rate of reaction

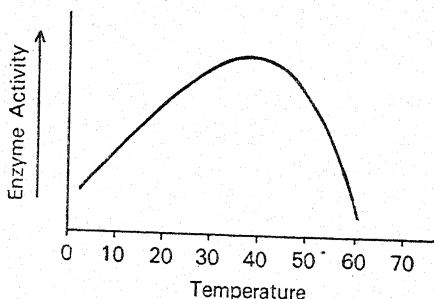


Fig. 3.3. Effect of temperature on enzyme activity.

is balanced by destruction of an isolated enzyme. At extremely low temperatures, the enzyme activity virtually ceases, but without any damage to the enzyme. Thus isolated enzymes may be preserved at temperatures at or below 0°C.

### (2) Hydrogen Ion Concentration

Like temperature, extreme variations in the pH may also destroy enzymes. Each enzyme exhibits maximum activity at an optimum pH and deviation from the optimum may result in reduced enzyme-activity. Haldane (1930) investigated the pH relation of a large number of enzymes, and observed that a majority of them had their pH optima between 4 and 8. Media with pH between these limits, support the growth of most fungi, but the influence of pH of the

external media on the internal pH of cells is yet to be understood. Relationship between enzyme activity and pH is shown in Fig. 3.4.

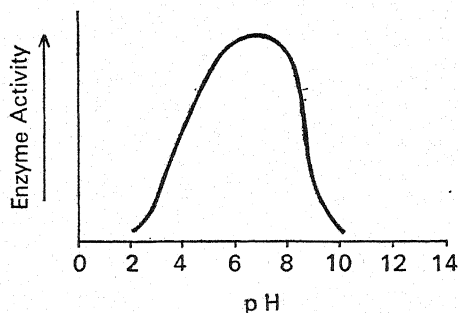


Fig. 3.4. Effect of pH on enzyme activity.

### (3) Enzyme/Substrate/Product Ratio

The rate of enzyme-catalyzed reaction depends upon the concentration of all the three components, viz., the substrate, enzyme and the end-product. Within certain limits, there exists a linear relationship between enzyme concentration and enzyme-activity. Increased substrate concentration also causes an initial enhancement in the activity of the enzyme (Fig. 3.5). However any further increase in the amount of the substrate causes a decrease in the rate of the reaction. Like other chemical reactions, sooner or later the enzyme-catalyzed reaction also attains an equilibrium when no further transformation of the substrate into the product occurs. Establishment of an equilibrium is, however, prevented if the product is not allowed to accumulate beyond a specified level. Otherwise, the increasing amount of

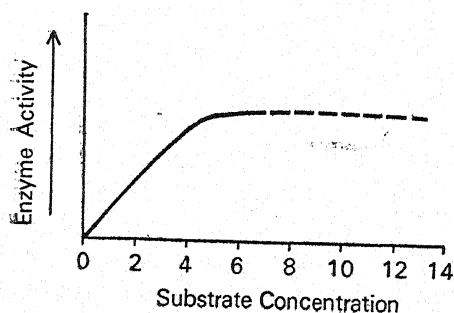


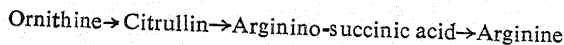
Fig. 3.5. Effect of substrate concentration on enzyme activity.

the product, in relation to the substrate concentration, exerts a mounting inhibitory effect which may even lead to the reversal of the

reaction. Such controlling effects of the end-product on enzymatic reactions and their role in the overall regulation of the cellular metabolism has been extensively studied in bacteria. The accumulated end-product has been found to exert its controlling-effect over an enzyme-reaction, principally through two different mechanisms, viz. (a) feedback inhibition, and (b) feedback repression.

(a) *Feedback inhibition*. This kind of inhibition was discovered in *Escherichia coli* by Novick and Szilard (1954). They observed that accumulation of tryptophan exerted an inhibitory effect upon the synthesis of its precursor. Such inhibitory effects may also be produced, if the performed product of the reaction is added to the medium from outside. Roberts *et al.*, (1955) reported that addition of amino acids to the medium inhibited their endogenous production in yeast and *E. coli*. The only other fungus, where the feedback inhibition has been studied to some extent is *Neurospora crassa*, although in bacteria, and particularly in *E. coli* (Umbarger, 1961 a, b) investigations on this aspect have been extended to several biosynthetic reactions. In *Neurospora crassa*, addition of tryptophan inhibited the synthesis of anthranilic acid (Lester, 1963; Matchett and DeMoss, 1963).

Similarly, Bechet *et al.* (1962) reported that arginine inhibited the enzyme ornithine transcarbamylase, which catalyzed the synthesis of citrullin from ornithine and carbamyl phosphate in the following reaction-sequence:



The feedback inhibition causes a reversible impairment in the activity of the enzyme, possibly without interacting with the active site of the enzyme (Monod *et al.*, 1963). The characteristic feature of this kind of inhibition is that inhibitory effect of the end-product inactivates an enzyme responsible for an early step of the reaction-sequence (Fig. 3.6). Thus it provides to the cell a means to check the formation of an end-product as soon as it begins to accumulate. The enzyme is, however, set free to restart the synthesis of the end-product as and when the latter gets depleted.

(b) *Feedback repression*. In feedback repression, the end-product of a reaction-chain suppresses the formation of one or all (co-ordinate repression) the concerned enzymes. Thus, in this mechanism the very synthesis of the enzymes, and not only their function, is inhibited. It is obvious that this mechanism becomes operative gradually, because the preexisting enzyme molecule will continue to

produce the end-product, until they become completely non-functional. Of course, repression has a distinct advantage as a long-term device, namely, that by completely preventing the synthesis of the enzymes it proves economical to the cell.

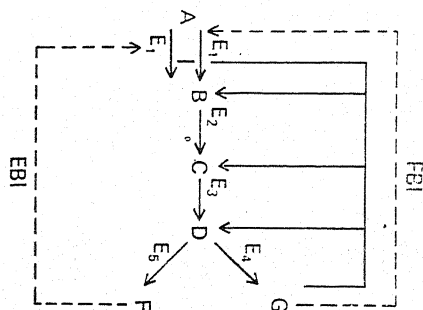


Fig. 3.6. Showing feedback inhibition and repression of enzyme activity. A—D, intermediates of a synthetic pathway. G & F, alternative products. E<sub>1</sub> and E<sub>1</sub>, isoenzymes for the step AB; E<sub>2</sub>—E<sub>5</sub>, enzymes active at subsequent steps. FBI and EBI, showing feedback inhibition of the enzymes E<sub>1</sub> and E<sub>1</sub>, respectively. FBR, showing feedback repression of enzymes E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub>.

Both these mechanisms may function together in the same pathway, and due to the same ultimate effect the two are hard to distinguish. Tryptophan, for example not only causes a feedback inhibition in

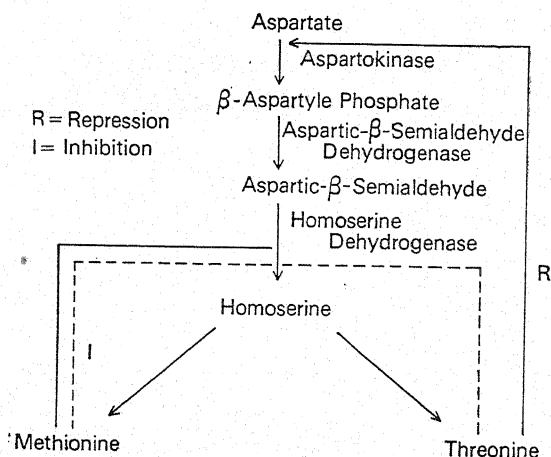


Fig. 3.7. Showing simultaneous inhibition and repression of enzyme activity.

*Neurospora*, but also effects a repression of the enzyme tryptophan synthetase in some auxotrophic mutants (Matchett and DeMoss,

1962). In yeast, methionine and threonine inhibit the enzyme homoserine dehydrogenase (Karassevitch and De Robichon Szulmajster, 1963) both by feedback inhibition as well as repression (Fig. 3.7). Methionine causes both inhibition and repression of the same enzyme, while threonine represses the enzyme aspartokinase and inhibits the homoserine dehydrogenase. Yet another example of coexistence of the two mechanisms is reported from *Neurospora* (Donachie, 1964), the enzyme affected being aspartyl carbamyl transferase.

Repression of some enzymes have also been reported to be caused by substances other than the end-products. Designated as catabolite repression (Magasanik, 1961), it is caused by some metabolite not connected with the enzyme-reaction. An example of catabolite repression is the repressing action of glucose on formation of some enzymes. Such "glucose-effect" in yeast is reported to inhibit the synthesis of various TCA cycle enzymes, including succinic and isocitric dehydrogenases (MacQuillan and Halvorson, 1962 b) besides the  $\beta$ -glucosidase.

Inhibition and repression mechanisms together with the induction of enzymes are such phenomena that obviously exert profound impact on regulation of the metabolic activities of Fungi.

#### (4) Chemical Agents

A variety of chemical agents are known which either function as inhibitor or activator of enzymes. Some of the chemicals exert opposite effects upon different enzymes, activating one but inhibiting the other.

**Activators.** The metallic ions are well known activators of enzymes. As many as fifteen metal ions, including  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$ ,  $\text{CS}^+$ ,  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Cd}^{++}$ ,  $\text{Cr}^{+++}$ ,  $\text{Cu}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$  and  $\text{Al}^{+++}$  activate one or more enzymes. Molybdenum is also known to activate certain enzymes, e.g. nitrate reductase of *Neurospora crassa*. However, it is better to treat these metal ions as essential component of enzymes than as activators.

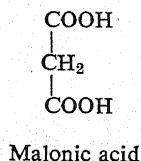
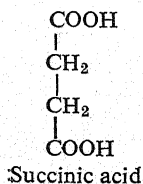
That anions may also function as activator was first shown by Cole (1904). Chloride is an excellent activator of  $\alpha$ -amylase of animal origin. Other monovalent anions may also exert some stimulating effect on this enzyme. However, the enzyme fumarate hydratase was shown to be activated by divalent and trivalent anions only (Mann and Wolf, 1930). But most of the enzymes are little affected by anions at ordinary concentrations.

The activating effects of the cations and anions are somewhat different. Effects of anions are unspecific, and the enzyme may remain somewhat active even without the electrolytes. Moreover, any anion will have some effect. On the contrary, the requirement of a cation is fairly specific and in some cases only one particular cation is functional. Moreover, the enzyme is completely inactive in want of that particular cation. Fumarate hydratase is the example of the first kind and is activated by a wide range of anions, whereas the inorganic pyrophosphatase specifically requires  $Mg^{++}$  ions and none else will serve the purpose.

Some other chemical agents have also been reported as activator of enzymes. Hydrogen sulfide, glutathione and other thiol compounds activate certain plant proteinases, *e.g.* papain and bromelin.

*Inhibitors.* To this class of compounds generally those agents are included, which cause a reversible or irreversible inhibition of enzymes by some chemical action. Some of the inhibitors compete with the substrate for an active centre on the enzyme surface and thus tend to drive away the substrate. These are called competitive inhibitors and ~~their effect~~ may be nullified in high substrate concentration. The non-competitive type of inhibitors do not affect the combination of substrate with the enzyme, rather the enzyme may combine with both inhibitor and the substrate at the same time forming an EIS complex. The EIS complex may not break-down at all or may break-down slowly, thus inhibiting the enzyme activity. In some other instances the non-competitive inhibitor combines with the metallic co-factors and thus render the enzyme inactive.

The competitive inhibitors are somewhat similar in structure to the substrate, and therefore are able to compete with the substrate for attachment to the enzyme. The competitive inhibitors are generally specific for a single enzyme, and a large number of such compounds are included in the literature. The classical example is malonate which is a strong competitive inhibitor of succinate dehydrogenase owing chiefly to a close similarity in the structure of succinic and malonic acids:





Pyrophosphate also inhibits succinic dehydrogenase which again is obviously due to the presence of two acid groups in its molecule. Massey (1953) found that fumarate hydratase was inhibited by the salts of almost all the di- or tricarboxylic acids tested by him, including malonate. But monocarboxylic acids did not cause any inhibition. In fact, several TCA cycle enzymes, which have di- or tricarboxylic acids as their substrate, are now known to be inhibited by a range of such acids. The competitive inhibitors show stereochemical specificity similar to that shown by the substrate. For example, arginase is inhibited by L-leucine but not by D-leucine.

All other enzyme-inhibitors, which do not compete with the substrate, form the large group of non-competitive inhibitors. These include a variety of chemical agents, like respiratory poisons reagents for thiol groups, heavy metals etc. Respiratory poisons are inhibitors of cytochrome oxidase system, and act by forming metal-complexes with their metallic co-factors. The cytochrome oxidases are metalloproteins and are generally catalyzed by iron and copper. Inhibitors of this group, including cyanide,  $H_2S$ , azide etc. generally inactivate all those enzymes which contain iron or copper.

Certain compounds with affinity for thiol groups act as inhibitors of  $-SH$  enzymes. Examples of such inhibitors are iodoacetate, ethyl iodoacetate, iodoacetamide, p-chloromercuribenzoate, oxidized glutathione, Iodosobenzoate, etc., all attacking the  $-SH$  group but in some what different manner.

Most of the enzymes are inhibited by salts of heavy metals, like Ag, Cu, Hg and Pb. At higher concentration they denature the enzymes, and the proteins in general. At lower concentration, these metals inactivate a few enzymes, but their exact mode of action is not known. Suggestions regarding their activity through action on thiol groups, carboxyl groups etc. have been put forward but no generalisation is possible at present.

## CARBON SOURCES AND THEIR UTILIZATION

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Living organisms are known to utilize about forty elements, among which carbon plays the key role. As a component of both structural and functional cell-constituents, carbon comprises about fifty per cent of the total mycelial dry-weight in fungi. A multitude of organic constituent of fungal cell, like carbohydrates, proteins, nucleic acids, enzymes etc. are all made up of carbon. Practically, all the important components of cell-wall, like cellulose, chitin, and pectic substances contain carbon in varying form and concentration, and thus provide the structural frame-work of the fungal cell. In their functional role, carbon compounds are still more significant, because fungi, being chemotrophs, obtain all their energy-requirements from catabolic degradation of one or other carbon containing ingredients of the cell.

Fungi exhibit carbon heterotrophy and obtain their carbon-requirement from various organic sources, although in a few cases utilization of inorganic carbon in the form of  $\text{CO}_2$  has also been reported but not as the sole source. A variety of organic compounds are utilized by fungi, and the nature of the organism largely determines the range of substrate. A massive literature has accumulated on carbon-nutrition of fungi. Much of this account is of repetitive nature, but it has been helpful in deriving certain general conclusions. For example, the monosaccharides have generally been reported to be more easily utilizable sources than the oligo- or the poly-saccharides. Though, interesting inferences have been derived on the basis of laboratory experiments, yet a wide gap still exists between the facts and the conclusions. This is mainly because the nature of a carbon compound available under natural conditions is sometimes quite different from the one on which the fungi have to feed under controlled laboratory conditions. The crude form in which various compounds exist in nature are generally not so readily utilized under the cultural conditions. This obviously reflects that the efficiency of the

organism is much more put to challenge under natural conditions than under the laboratory set up. The mere fact that there is an enormous fungal flora comprising the organic world is a proof of their efficiency to derive food from different natural substrates.

Much of the conclusions that have been derived in the laboratories are either based on measurement of radial growth on agar plates, or on determining the dry weight of the mycelial harvest after a certain incubation period. Both these methods suffer with certain lacuna and the inferences derived are often a subject of controversy. Periodical determination of the left-over food, and the pH of the medium also play a decisive role. But majority of the investigators have not taken precaution to evaluate the role of these aspects. Numerous experimental shortcomings are also sometimes responsible for erroneous conclusions, which have been pin-pointed by Cochrane (1958).

The bulk of work on carbon nutrition has been so tremendous that it is not possible to comprehend all the literature within the scope of this book. Endeavour has, however, been made to give the general conclusions and the points of contradictions. Many a times the variations have been attributed to the change of the laboratory set-up or to genetic make-up of the organism.

A wide variety of carbon sources including carbohydrates, organic acids and amino acids, alongwith their derivatives, as well as some polycyclic compounds and alkaloids are used by fungi. Perlman (1965) has given a comprehensive list of such compounds. Many fungal species are able to thrive on different kinds of aliphatic hydrocarbons, also. In view of their efficiency to utilize a large number of carbon-sources, Perlman (1965) has pointed out, that the only possible exceptions to fungal metabolism of carbon seem to be some plastics, flourine containing compounds and biologically non-degradable detergents. However the carbohydrates and some of their derivatives have generally been found to be the most widely utilizable sources of carbon, both in nature and under laboratory conditions. In view of the large variety and complex structure of these compounds, a brief account of their chemical make-up seems necessary, prior to any discussion on the role and utility of individual carbon-sources in fungal nutrition.

## CHEMICAL NATURE OF CARBON SOURCES

### (I) CARBOHYDRATES

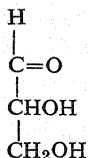
The carbohydrates are organic compounds consisting of carbon, hydrogen and oxygen, the later two in a ratio of 2 : 1 as in water. Chemically, they are defined as aldehyde or ketone-derivatives of higher polyhydric (those with more than one OH group) alcohols, or as compounds which yield these derivatives on hydrolysis. The simplest carbohydrate may be represented by the general formula,  $(\text{CH}_2\text{O})_n$ .

Carbohydrates have been classified into (i) monosaccharides, having a single sugar unit; (ii) disaccharides, having two sugar units joined together by what is called a glycosidic linkage; and (iii) polysaccharides, or 'multiple sugar', having several or many sugar units joined together into branched or unbranched polymeric chain. Some smaller polysaccharides with known number of sugar residues are generally categorised separately as oligosaccharides.

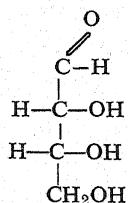
#### Monosaccharides

These compounds are also popularly referred as simple sugars and are sweet in taste and soluble in water. They have a general formula  $\text{C}_n(\text{H}_2\text{O})_n$ . They possess a free (or "Potentially free") aldehyde (-CHO) or ketone (-CO-) group, besides the primary (-CH<sub>2</sub>OH) and secondary (-CHOH-) alcohol groups. In a monosaccharide carbon unit, the aldehyde and primary alcohol groups are attached on the two extremities, while the ketone group is located on the second carbon atom.

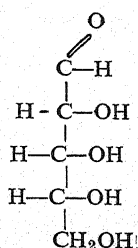
Classification of these sugars is based on the number of carbon atoms present, and the functional group involved. As per the former criterion, monosaccharides may be a triose if the chain has three carbon atoms, a tetrose with a chain of four carbon atoms, a pentose, five carbons and hexoses with six carbons etc.



D-Glyceraldehyde  
(Triose)



D-Erythrose  
(Tetrose)



D-Ribose.  
(Pentose)

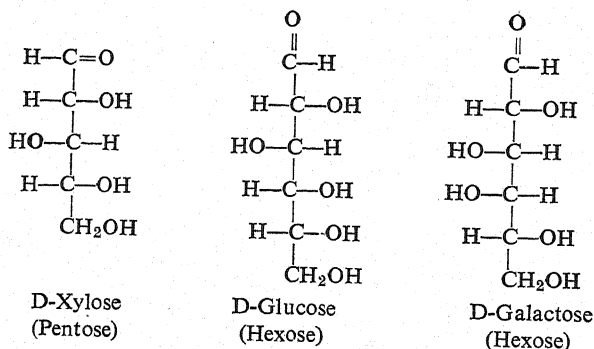


Fig. 4.1. (a) Structure of some monosaccharides.

On the basis of their functional group, a simple sugar may be an aldose or a ketose, e.g., glucose is an aldohexose, while fructose a ketohexose:

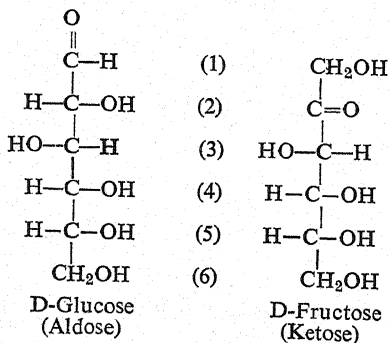


Fig. 4.1. (b) Fischer structures of D-glucose and D-fructose. Numbers in parenthesis denote the system employed for numbering the carbon-atoms.

## ISOMERISM

Compounds with identical composition and the same molecular weight but of different structures are called *isomers*, and the phenomenon is known as *isomerism*. Simple sugars exhibit two different types of isomerism, (1) Spatial isomerism, and (2) Optical isomerism.

### (1) Spatial Isomerism

This type of isomerism is due to the characteristic molecular structure of these sugars. Number of such spatial isomers depends upon the number of\* asymmetrical carbon atoms in the molecule.

\*A carbon atom to which four atoms or groups of atoms are attached is said to be asymmetrical.

Thus, the simplest monosaccharides the trioses have the minimum number of isomers, because it has only one asymmetric carbon atom, *i.e.* carbon two (Fig. 4.1). Moreover, trioses exhibit only one type of spatial isomerism, *viz.* enatiomorphic isomerism while tetroses, pentoses, and hexoses show the following two types.

(A) *Enatiomorphic isomerism.* These isomers, also called enatiomorphs show very little difference from each other. They are simply mirror-images of one another, and hence represent two forms of the same compound. These isomers occur in pairs and possess the same physical properties. However, fungi are able to distinguish between enatiomorphs, as, was first shown by Pasteur (1860) with *Penicillium glaucum*. A fungus may utilize one enatiomorph, while rejecting the other. This mirror-image type of isomerism is indicated by the use of Roman capitals D or L preceding the name of the sugar, *e.g.* D-glucose, L-glucose etc.

A sugar is designated as D- or L- depending upon the orientation of the hydroxyl group attached to the highest numbered asymmetrical carbon atom in the chain. Thus, in a hexose molecule, it is the asymmetrical carbon 5 which matters while in a pentose it is the carbon 4, and so on. In a D-form, the hydroxyl group attached to this particular asymmetric carbon is represented, in the Fischer formula, on the right side of the carbon chain. Obviously, the same hydroxyl group in a L-form sugar, will be shown on the left side of the carbon-chain. Thus, the over-all structures of D and L forms are the mirror-images of each other as shown in the figure:

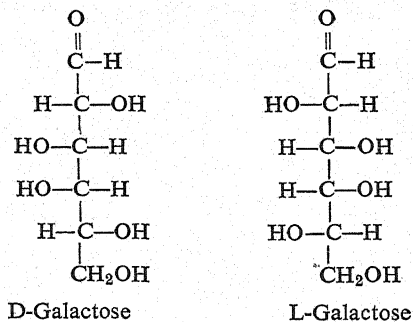


Fig. 4.2. D- and L-enatiomorphs of galactose

(B) *Diastereo isomerism.* Compounds showing this type of isomerism are not the mirror images of one another, and differ in their physical, chemical and biological properties. They contain the same functional groups but in different orders. Therefore, diastereoisomers

represent different compounds and are designated by different names. For example glucose, mannose and galactose are diastereoisomers as are D-fructose and L-sorbose. Formation of such types of isomers is due to the different arrangements of hydrogen and hydroxyl groups around the asymmetrical carbons. The structures are given below:

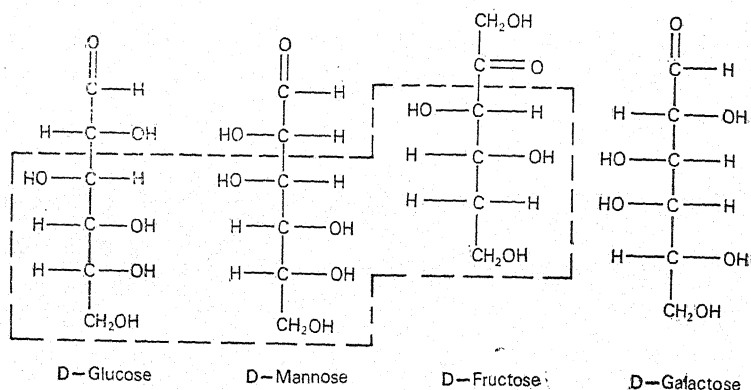


Fig. 4.3. Diastereoisomers of glucose, mannose, fructose and galactose. Area of similarity between D-glucose, D-mannose, and D-fructose, shown by dotted lines.

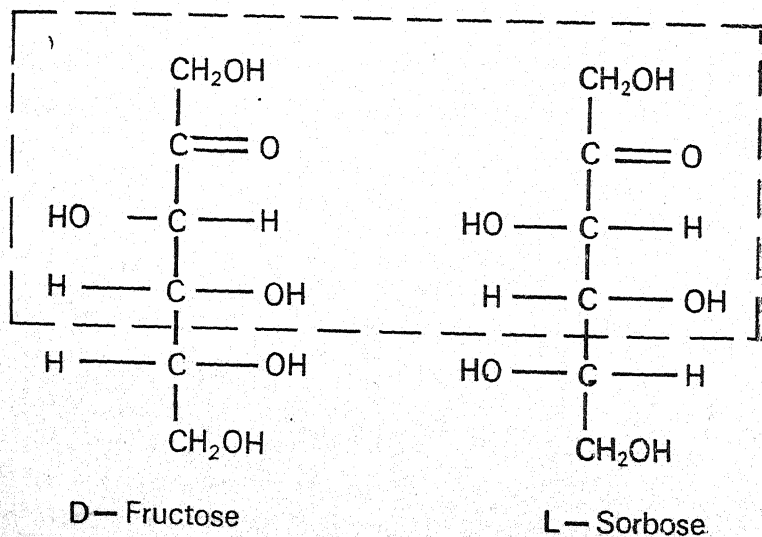


Fig. 4.4. Diastereoisomers of D-fructose and L-Sorbose.



Two sugars, which differ from one another in configuration around a single asymmetrical carbon atom are called *epimers*. Glucose and galactose are epimers with respect to carbon 4, while mannose and glucose are epimeric around carbon 2.

## (2) Optical Isomerism

Compound showing optical isomerism may either be dextro-rotatory or levorotatory, *i.e.* in pure aqueous solution it may cause a rotation in the plane of polarized light either to the right or to the left side, respectively. The dextrorotatory and levorotatory isomers are indicated respectively by + and - signs in parentheses, prior to the name of the compound, *e.g.* *D* (+) - glyceraldehyde and *D* (-) - glyceraldehyde. This optical-rotation type of isomerism should not be confused with enatiomorphic isomerism denoted by *D* and *L*, particularly because in the older literature, optical isomers were used to be indicated by small italic letters *d* or *l*. In fact, the former is independent of the latter. Some *D*-isomers may exhibit dextro- or levo-rotatory powers and are accordingly represented as *D* (+) and *D* (-). Similarly, *L*-isomers may also be levo- *L*[-] or dextro [*L*(+)] rotatory types.

## CYCLIC FORMS OF SUGARS

Aldehydes and ketones have characteristics to form hemiacetals and hemiketals respectively. This is achieved by an addition reaction at carbonyl group. Sugars, which typically contain an aldehyde or a ketone group also show such reaction. In an aldose sugar like glucose, addition of the aldehyde group takes place with an alcohol group attached to carbon 5. Thus, the glucose molecule attains a cyclic form containing a 5-carbon-1-oxygen-membered ring, similar to that of a pyran (a six membered ring heterocyclic compound, containing an oxygen atom). In this form, glucose is called a pyranose sugar. Likewise, a ketose, *e.g.* fructose, forms a hemiketal due to addition of the ketone with an alcohol group attached to carbon 4, and thus forms a 4-carbon-1-oxygen ring, known as furanose. The pyranose and furanose rings of glucose and fructose are represented in Fig. 4.5.

The pyranose or furanose rings shown above are marked either as  $\alpha$  or  $\beta$ . This is again a kind of isomerism exhibited by sugars. It is clear from Fig. 4.5 that with the formation of a cyclic ring, the carbon 1 in the glucose molecule and carbon 2 in the fructose molecule

become asymmetric and may lead to structural isomerism. Such isomeric pairs, as  $\alpha$  and  $\beta$ -D-glucoses are called anomers, and the asymmetric carbon 1 (carbon 2 in case of fructose) is called anomeric carbon atom. If the hydroxyl group attached to the hemiacetal or hemiketal carbon has the same orientation as the hydroxyl group attached to carbon-5, the ring represents in  $\alpha$ -isomer. However, if the reverse condition is true, it is a  $\beta$  form.

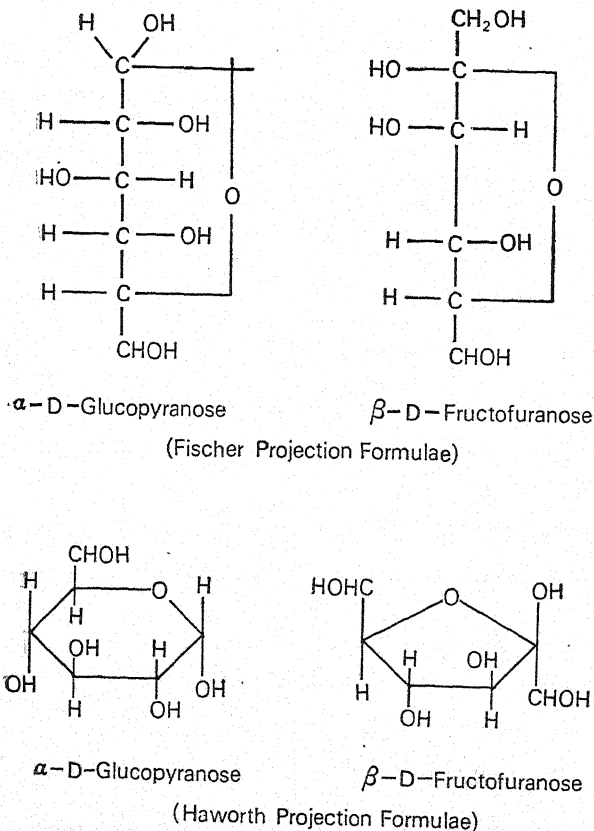


Fig. 4.5. Fischer projection and Haworth projection formula.

However, all these four ring-types viz. pyranose, furanose, and their  $\alpha/\beta$  forms are interchangeable. Actually any sugar in solution contains all the possible forms in a freely reversible equilibrium. For example, an aqueous solution of D-glucose actually represents an equilibrium mixture of five isomeric compounds as shown in Fig. 4.6.

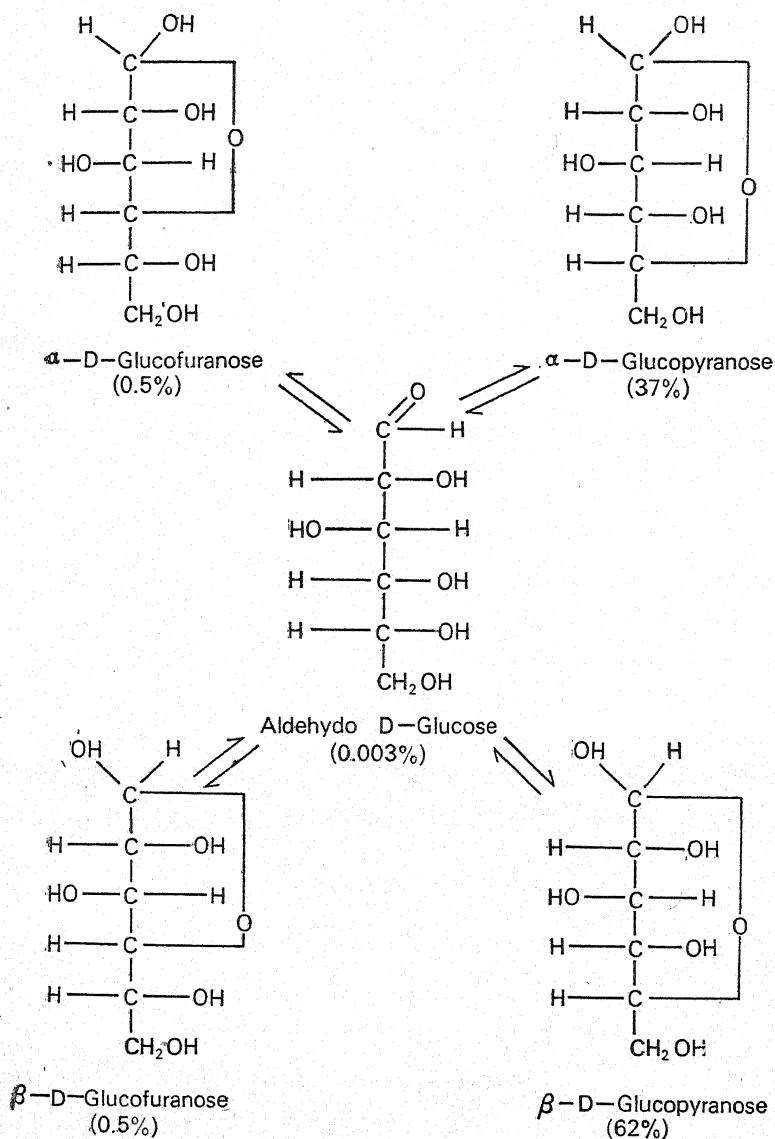
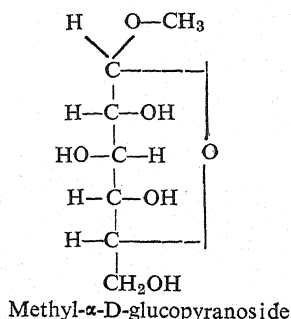


Fig. 4.6. Glucose isomers in aqueous solution at neutral pH.

## Glycosides



The ring form of all sugars is a hemiacetal and therefore they may react with another alcohol and form an acetal. All such acetal compounds are placed in a general class, known as glycosides, and specific glycosides are designated after the name of the sugar involved, *e.g.* glucoside, galactoside, fructoside etc. Like sugars, the glycosides may also have pyranose or furanose ring structures as well as their  $\alpha$  and  $\beta$  forms. However, unlike the hemiacetals, the  $\alpha$  and  $\beta$  glycosides are individually stable with respect to reciprocal transformations.

## Disaccharides

If the alcohol employed in a glycosidic linkage is a component of another sugar molecule, the product is a disaccharide. Structures of some common examples of disaccharides, like maltose, cellobiose, trehalose, lactose, melibiose, and sucrose are described below:

(a) *Maltose* (Two glucoses;  $\alpha$ -1, 4-glucoside linkage). From the structure of maltose it is obvious that one of the sugar units still possesses a free hemiacetal form. Therefore, maltose in a solution will comprise three different forms of molecules *viz.*  $\alpha$ -,  $\beta$ - and aldehyde, in a state of equilibrium. This disaccharide is obtained as an intermediate product during the digestion of starch to glucose.

(b) *Cellobiose* (Two glucoses;  $\beta$ -1, 4-glucoside linkage). This disaccharide is the repeating unit in cellulose, and differs from maltose only in the nature of the glucosidic bonds.

(c) *Trehalose* (Two glucoses;  $\alpha$ -1, 1-glucoside linkage). Trehalose is also made up of glucose moieties, which are held together by  $\alpha$ -glucosidic linkages. Unlike maltose, it is a non-reducing sugar. This is possibly the only disaccharide to be assimilated by fungi in sufficient quantity.

(d) *Sucrose* (Glucose plus fructose;  $\alpha$ -D-glucopyranosyl-1-2- $\beta$ -D-fructofuranoside). In a sucrose molecule both the carbonyl groups

are involved in the formation of glycoside linkage. Therefore, only one form of sucrose exists. Sucrose is designated as a non-reducing disaccharide, because in absence of any free aldehyde group in its molecule, it is not able to reduce Fehling's or Benedict's solution etc. However, maltose as well as lactose are categorised as reducing sugars due to their reducing abilities of Benedict's solution etc., owing to the presence of a free aldehyde group in their molecules.

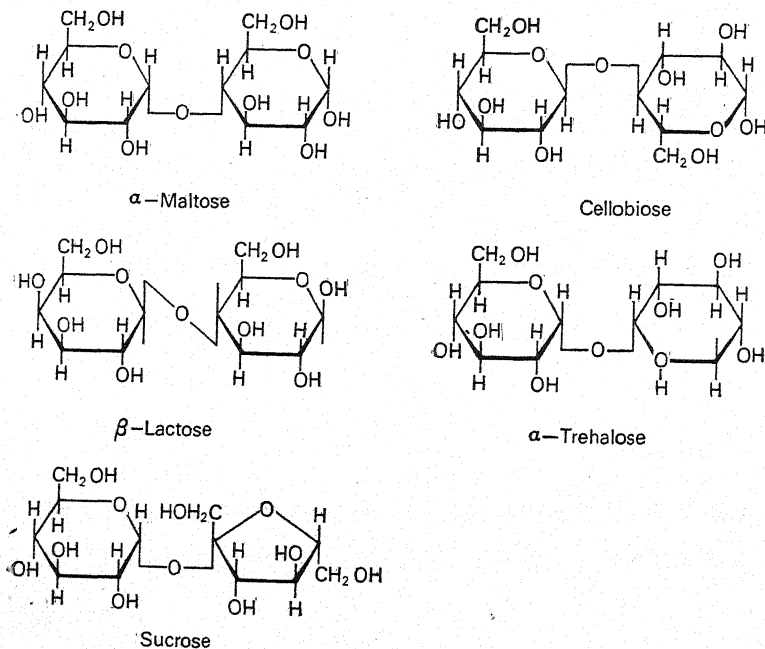


Fig. 4.7. Structural formula of some disaccharides.

(e) *Lactose* (Glucose + galactose;  $\beta$ -1, 4-galactoside linkage). Lactose, the milk sugar is a  $\beta$ -glycoside consisting of one each of glucose and galactose units and upon hydrolysis by acids or lactase yields an equimolar mixture of galactose and glucose.

(f) *Melibiose* (D-glucose + D-galactose;  $\alpha$ -1, 6-galactoside linkage). This disaccharide is an  $\alpha$ -glycoside consisting of a glucose and galactose units joined by  $\alpha$ -1, 6-linkage (Fig. 4.8). Thus it differs from lactose only in the linkage, otherwise the monosaccharide moities are the same in both.

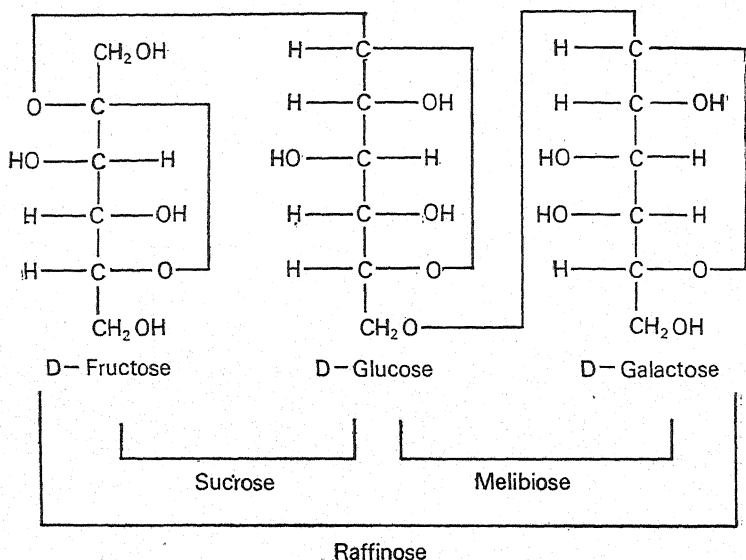


Fig. 4.8. Structural formula of Raffinose.

### Oligosaccharides

Oligosaccharides (oligo=few) consist of two to ten monosaccharide moieties and upon hydrolysis yield monosaccharides. However, sometimes disaccharides are also treated under oligosaccharides, besides the naturally occurring trisaccharides and tetrasaccharides etc, Raffinose, gentianose, etc. are the examples of trisaccharides, while stachyose is a tetrasaccharide which yields after hydrolysis glucose, fructose and two molecules of galactose.

### Polysaccharides

These are compounds of polymeric structure containing a large number of monosaccharide units. When all these units are of the same sugar, the polysaccharide is designated as homopoly-saccharide, while those comprising two or more different types of sugar units are called heteropolysaccharides. Common examples of naturally occurring polysaccharides are cellulose, starch, pectin, glycogen, etc. Brief description of the more important ones are outlined below:

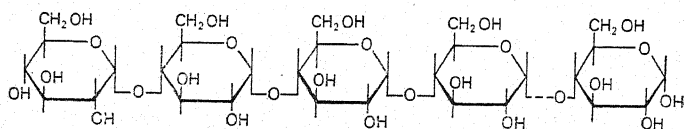
#### Starch

Starch is a compound of high molecular weight and is a polymer of D-glucose. It is generally found as a storage compound in plants and

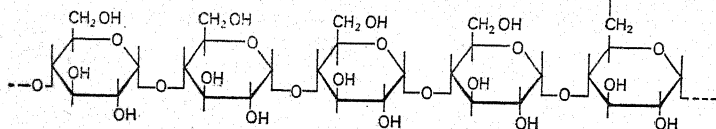
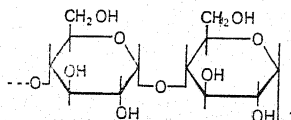
are stored as insoluble grains. Starch grains consist of two different polysaccharides having distinct properties.

(i) *Amylose*. This is a polysaccharide comprising a long unbranched chain of glucose units joined by  $\alpha$ -1, 4-glucoside linkage. These long chains readily come to lie in proximity and are then held together by hydrogen bonding between the hydroxyl groups. The resulting large structures are quite compact and are insoluble in water, unless the temperature is high enough to break the hydrogen bonds. This is generally achieved by boiling.

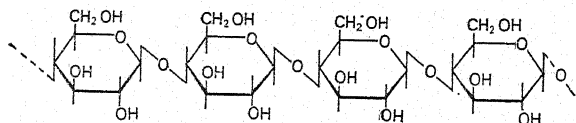
(ii) *Amylopectin*. The basic chain of this compound is quite similar to that of amylose and is made up of  $\alpha$ -1, 4-glucoside linkages. However, this compound possesses a number of side-chains also composed of  $\alpha$ -1, 6-glucoside linkages. On account of this branched structure the chains are not able to firmly adhere to each other. Therefore, the amylopectins are reasonably soluble in warm water.



Part of an amylose chain



Part of an amylopectin chain



Part of cellulose chain

Fig. 4.9. Structural formula of some polysaccharides.

### Glycogen

These are storage compounds of animals and fungi. They have



essentially the same structure as the amylopectin, except that the branched chains are much more extensive in glycogen. Therefore, these compound are still more soluble and are brought to suspension even in cold water.

### Cellulose

A major constituent of plant cell-wall, cellulose has a general structure similar to amylose. However in cellulose the glucose units are held together by  $\beta$ -1, 4-glucoside linkages. The cellulose macromolecules are composed of much more longer chains (1 to  $3\mu$  long with 2,000-10,000 glucose units). Due to their unbranched nature many such chains are firmly held together. This makes cellulose chains extremely insoluble and compact, properties so useful for them in view of their roles as building materials. Most of the microfibrils of primary and secondary walls of plant cells are composed of long cellulose chains. Preston *et al.* (1948) and Frey-Wyssling *et al.* (1948) have discussed the details of the composition of cellulose chains into microfibrils.

### Hemicelluloses

These are polymers of various monosaccharides or their derivatives. Some of the common hemicelluloses are xylans, mannans, galactans and glucans, in which D-xylose, D-mannose, D-galactose, and D-glucose predominate respectively. Some of these compounds have more than one type of monosaccharides abundant in their chains and are accordingly designated, *e.g.* glucomannans, galactomannans, arabogalactans etc. Xylans are probably the most important hemicellulose in higher plants and have a branched chain structure, with xylose in abundance and arabinose and gluconic acid in smaller amounts. In plant cell-wall hemicelluloses are perhaps distributed in an amorphous state between the cellulose micro-fibrils.

### Pectins

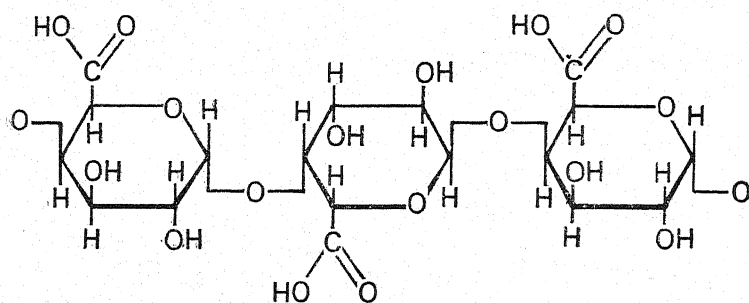
These are the polymers of galacturonic acids and are a major constituent of plant cell-wall. Generally three kinds of pectic substances have been recognised on the basis of their chain length and degree of their methyl esterification:

(i) *Pectic acid*. It possesses an unbranched chain of D-galacturonic acid units held together by  $\alpha$ -1, 4 linkages (Fig. 4.11). Pectic acid chain contains about 100 galacturonic acid residues and their large

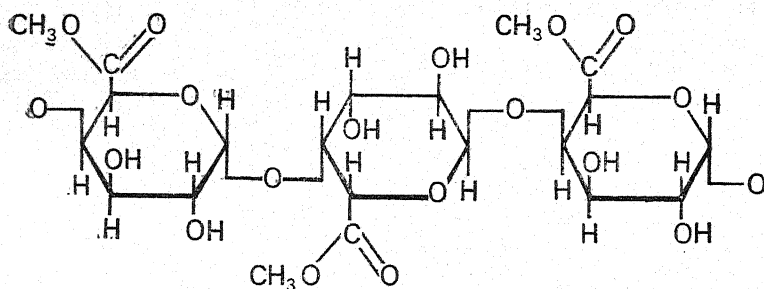
number of free carboxyls permit it to react with polyvalent cations like  $\text{Ca}^{++}$ . Some times arabinose and galactose remain associated with pectic acid, but their linkage-pattern is not yet known.

(ii) *Pectin or pectinic acid*. The general structure of pectin is similar to that of pectic acid, except that it has about 200 galacturonic acid units and that about 50-80% of its carboxyls are esterified with methyl groups. Both pectin and pectic acid are soluble in water and form colloidal suspension.

(iii) *Protopectin*. It has about 1000-2000 units of galacturonic acid residues whose carboxyl groups are also esterified. The larger molecules of protopectin are insoluble in water and are somewhat unstable.



A part of pectic acid chain



A part of pectinic acid chain

Fig. 4.10. Structure of pectic and pectinic acid.

## Chitin

These compounds are generally found as the structural components of some higher invertebrates; they also form a significant fraction of fungal cell-wall. These are straight chain polymers of N-acetylglucosamine units held together by  $\beta$ -1, 4-glycosidic bonds.

## Other Polysaccharides

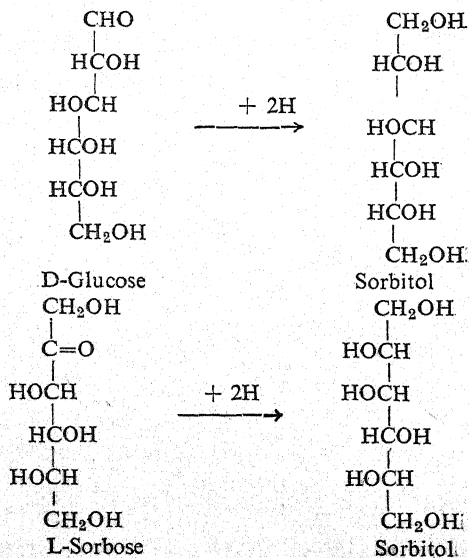
Various other plant products possess still more complex structures. Vegetable gums, mucilages etc. contain a variety of sugars and uronic acids. Agar-agar, comprise a complex mixture of D- and L-galactoses. Some other examples are, mannans—a mannose polymer, inulins—a fructose polymer etc.

## SUGAR DERIVATIVES

Carbohydrates also include some derivatives of sugars. Some of which are important with respect to fungal metabolism and nutrition. The more common ones are described below:

### Sugar Alcohols

When the aldehyde or ketone group of an aldose or ketose sugar is reduced to an alcoholic group, the resulting compound is a sugar alcohol. Sorbitol is a sugar alcohol obtained from D-glucose as well as L-sorbose. Similarly galactose on reduction gives rise to dulcitol while D-mannitol, is obtained from mannose. All these sugar alcohols are widely distributed in plant tissues. Their structures are shown below:



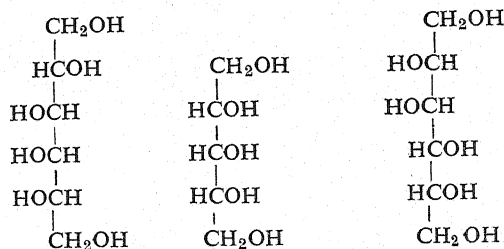


Fig. 4.11. Structure of some sugar alcohol.

### Sugar Acids

Aldoses are converted into sugar acids when either their primary alcohol group or the aldehyde group or both of them are oxidised into carboxyl groups. Depending upon the group which is oxidized into carboxyl, three kinds of sugar acids are identified:

- Uronic acid*. When the primary alcohol group is oxidized.
  - Glyconic acid*. When the aldehyde group is oxidized.
  - Glycaric acid*. When both the aldehyde as well as the primary alcohol groups are oxidized and thus, these are dicarboxylic acids.
- These acids are also called saccharic acids.

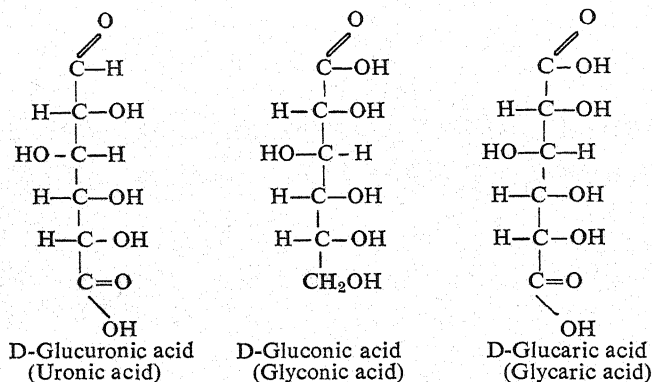


Fig. 4.12. Structure of some sugar acids.

### Amino Sugars

These are sugar derivatives, in which a hydroxyl group (generally attached to carbon 2) is replaced by an amino-group. Hexosamines, such as glucosamine, galactosamine, mannosamine etc. have been widely reported to occur in natural materials. In most of the cases an acetyl group is found attached to the nitrogen of the amino group.

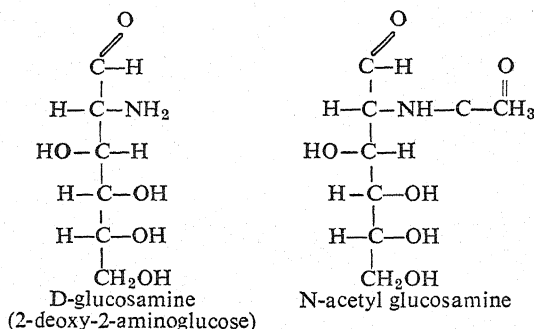
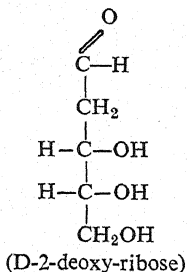


Fig. 4.13. Structure of amino sugars.

### Deoxy Sugar

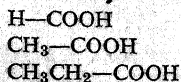
When a hydroxyl group of a sugar molecule is replaced by a hydrogen atom, a deoxy sugar is obtained. A common deoxy sugar is 2-deoxy-D-ribose, which is a constituent of nucleic acids found in all types of cells. Its structure may be elucidated as below:



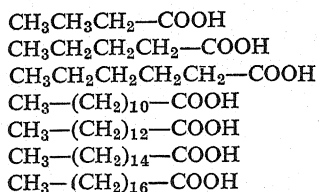
## (II) ORGANIC ACIDS

Apart from the carbohydrates and their various derivatives detailed above, organic acids also comprise an important source of carbon for many fungi. The presence of carboxyl group(s) ( $-\text{COOH}$ ) is a characteristic feature of all organic acids. When a single carboxyl group is present, the acid is designated as monocarboxylic acid, while those with two carboxyl groups are called dicarboxylic acids. Structures of only a few members of the two series will be illustrated here to serve our limited objective.

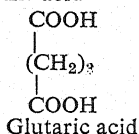
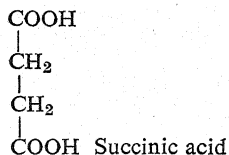
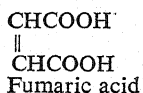
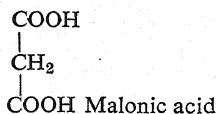
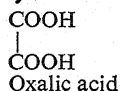
### Monocarboxylic Acids



Formic (methanoic) acid  
Acetic (ethanoic) acid  
Propionic (propanoic) acid

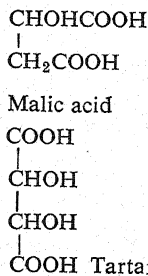
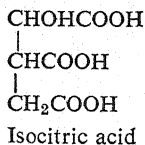
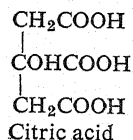
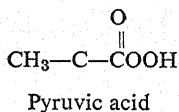


Butyric (butanoic) acid  
Valeric (pentanoic) acid  
Caproic (hexanoic) acid  
Lauric (dodecanoic) acid  
Myristic (tetradecanoic) acid  
Palmitic (hexadecanoic) acid  
Stearic (octadecanoic) acid

**Dicarboxylic acids**

Structure of some dicarboxylic acids.

However, some of the biologically important organic acids do not fall under these two categories. Citric acid is a tricarboxylic acid; pyruvic acid is a keto-acid, and contains a ketone group in addition to a carboxyl group and some others, like isocitric, malic and tartaric acids are termed as hydroxy acids, because they contain additional hydroxyl groups apart from those present in their carboxyl group. Structures of some of them are shown here:



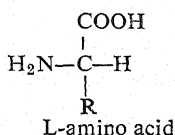
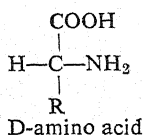
Structure of some other organic acids.

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## (III) AMINO ACIDS

These are the compounds which possess the properties of both acids and amines, owing to the presence of both a carboxyl ( $-\text{COOH}$ ) as well as amino ( $-\text{NH}_2$ ) group in their molecule. Amino acids are known to serve both as carbon and nitrogen sources of fungi and therefore structure of a few of these compounds are described here.

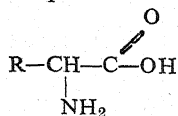
Like sugars, amino acids may also be D- or L-isomeric type (except glycine) owing to the position of the amino group in the molecule, as shown below:



In protein synthesis only L-amino acids are utilized, although D-isomers are also present in the cells.

Similarly, amino acids may also be either dextrorotatory (+) or levorotatory like the sugars, e.g. D- (+) -alanine, D- (–) -alanine.

Amino acids may be represented by a general formula,



and differ from one another mainly in composition of the side chain or R-group because the amino-group, carboxyl as well as the  $\alpha$ -carbon are common to all. Altogether seven groups of amino acids are recognised on this basis:

(1) *Simple amino acids*. (With no functional group attached to the side-chain).

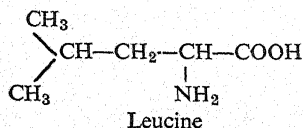
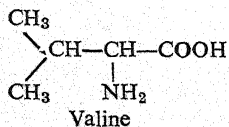
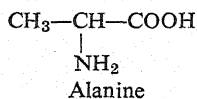
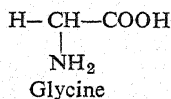


Fig. 4.14. Simple amino acids.

(2) *Hydroxy amino acids*. (With an alcohol group ( $-\text{OH}$ ) attached to the side-chain):

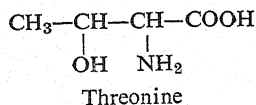
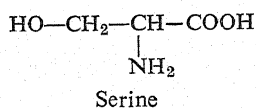


Fig. 4.15. Hydroxy amino acids.

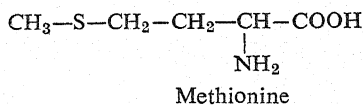
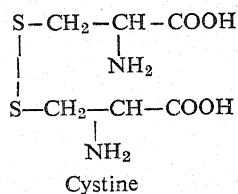
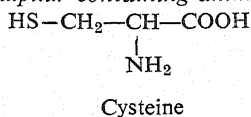
(3) *Sulphur containing amino acids.*

Fig. 4.16. Sulphur containing amino acids.

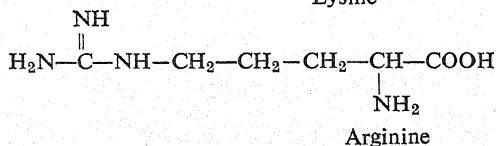
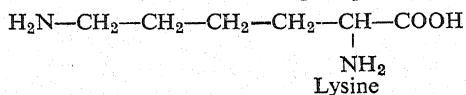
(4) *Basic amino acid.* (With a basic group in the side chain).

Fig. 4.17. Basic amino acids.

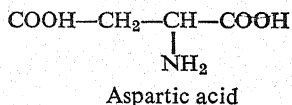
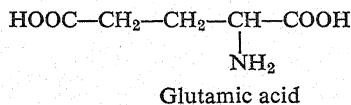
(5) *Acidic amino acid.* (With a carboxyl group in the side-chain).

Fig. 4.18. Acidic amino acids.

(6) *Heterocyclic amino acid.* (The side-chain with a ring, containing at least one atom other than carbon).



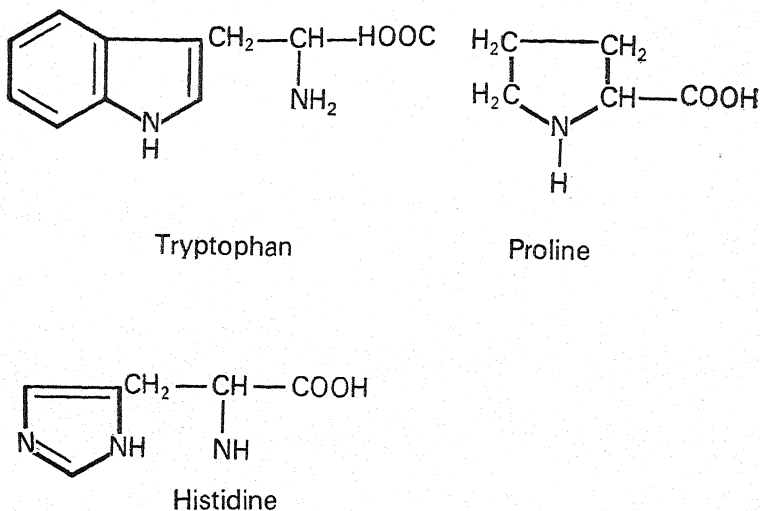


Fig. 4.19. Heterocyclic amino acids.

(7) *Aromatic amino acids.* (With an benzene-like aromatic ring in the side-chain):

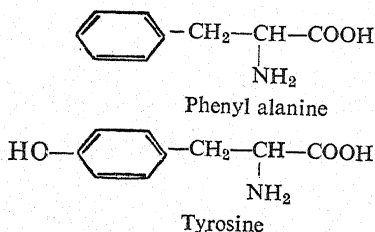


Fig. 4.20. Aromatic amino acids.

### Amides of Amino Acids

The carboxyl group attached to the side-chain of acidic amino acids may be replaced by an amino group to form amides as shown under:

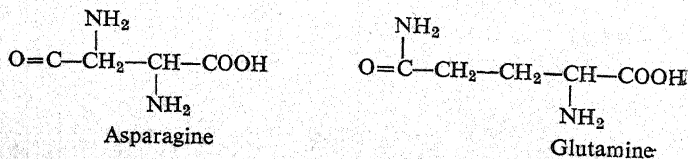


Fig. 4.21. Amides of amino acids.

## UTILIZATION OF CARBON SOURCES

**Monosaccharides**

Monosaccharides, usually, are easily assimilable form of carbohydrate, among which glucose has been reported to be the most efficient source of carbon and energy for most of the fungi. Some fungi like *Polychytrium aggregatum* (Ajello, 1948) exhibit exclusive choice for this sugar and fails to grow on other hexoses. Incapacity to use glucose is restricted to a few fungi only, like *Sphaeronema fimbriatum* (Weimer and Harter, 1921), *Fusarium lini* (Tochinai, 1926) *Leptomitius lacteus* (Schade, 1940), *Sclerotium rolfsii* and *Ozonium texanum*, var. *Parasiticum* (Grover and Chona, 1960), *Pythium aphanidermatum* (Kumar and Grover, 1967) and *Tieghemomyces parasiticus* (Barnett, 1970; Binder and Barnett, 1973), a biotrophic haustorial mycoparasite. However for another biotrophic mycoparasite, viz. *Gonatobotryum fuscum* glucose was the best carbon source (Calderone and Barnett, 1972). *Ustilago striiformis* was also unable to grow on glucose, unless incubated for 2-4 weeks (Cheo, 1949). Glucose is also a constituent of different fungal mycelia. Irani and Ganpathi (1960) reported that mycelium of *Penicillium chrysogenum* exhibited consistent presence of glucose, which they attributed to the fact that glucose is the end-product of various primary reactions, or it might be due to the break-down of polysaccharides as a result of starvation. Hasija and Wolf (1969) reported that in *Aspergillus niger* glucose was always present as a constituent of mycelium, irrespective of the carbon source.

It was recognised very early that glucose was not only a preferred carbon-source for many of the fungi, but it also interfered with the utilization of other carbohydrates. The earliest reports on this aspect were probably by Katz (1898) that amylase induction in *Penicillium*, and by Dienert (1900) that galactozymase activity in yeast were inhibited by glucose. The modern concept of this phenomenon which is now referred to as "glucose-effect" was developed by Epps and Gale (1942). They concluded that repression by glucose was due to its effect upon certain degradative enzymes, and was not a mere side-effect of acid production during fermentation. Magasanik (1961) has reviewed the literature and has renamed the phenomenon as catabolite-repression. In this phenomenon, glucose (or any other readily metabolized C-source) causes a reduction in the rate of synthesis of certain degradative enzymes. Since

then two more mechanisms in the control of carbohydrate utilization by glucose have been reported, viz. (i) transient repression (Boezi and Cowie, 1961; Paigen, 1966a; Moses and Prevost, 1966) and (ii) catabolite inhibition (Gaudy *et al.*, 1963; Stumm-zdliinger, 1966), which involves the inhibition of enzyme-action and not the formation and is a general phenomenon in the control of carbohydrate utilization (McGinnis and Paigen, 1969). A review on all these mechanisms along-with the names of fungi, where such repressive effects of glucose have been reported, has recently been presented by Paigen and Williams (1970).

Values of fructose, mannose, and galactose have been reported to be almost similar to glucose for *Stereum gausapatum* (Herrick, 1940) *Memnoniella echinulata* (Perlman, 1948), *Penicillium digitatum* (Fergus, 1952), *Chalara quercina* (Beckman *et al.*, 1953) *Colletotrichum inamdari* (Hasija, 1965), *Zygorhynchus moelleri*, *Z. Californiensis*, and *Z. macrocarpus* (Sarbhoy, 1965), *Alternaria tenuis* (Singh and Tandon, 1966), *Lophotrichus ampullus* (Kumar and Grover, 1967), *Alternaria citri*, *A. tenuis* and *Curvularia pallescens* (Hasija, 1968, 70), *Aspergillus niger* (Hasija and Wolf, 1969), *Pestalotia sapotae* and *P. versicolor* (Agarwal and Agnihotri, 1970) and *Curvularia* spp. (Singh and Tandon, 1970). Tandon (1963) presented the comparative values of these four hexoses for twelve leaf-spot fungi and observed that glucose and fructose were good carbon sources for all the species, while galactose was good or moderate for all but one species. Mannose, on the other hand, was a poor carbon source for as many as four species, and good or moderate for the remaining eight species.

Galactose, which differs from glucose, fructose and mannose in configuration around its fourth carbon atom has also been reported as unsatisfactory source for a number of fungi (Lilly and Barnett, 1951). *Melanospora destruens* (Hawker, 1939) does not make any growth on this sugar. Lilly and Barnett (1953) reported that, out of 57 fungi studied by them about 1/5th were unable to grow on D-galactose. *Chytriumyces aureus* and *C. hyalinus* exhibited poor or no response to galactose (Hasija and Miller, 1971). *Helminthosporium sativum* attains identical vegetative growth on glucose and galactose if incubation period is enhanced and asparagine is added to the medium. Such behaviour suggests the formation of induced enzymes. Roberts (1963) observed that a strain of *Aspergillus nidulans* possessed an inducible enzyme system which could oxidize D-galactose; D-fucose was, however, a poor inducer of that enzyme-system. Identical nutritive value of glucose and galactose in some species of

*Fusarium*, (Lilly and Barnett, 1953), of galactose and fructose in some species of *Phyllosticta* and *Pestalotia* (Tandon and Bilgrami 1957, 59) etc. suggest that structural configurations of these compounds do not play any decisive role in their utilization (Cf. Lilly and Barnett 1953). Cantino (1949), however, suggested that the assimilation of sugars by fungi depends upon their chemical structure as well as the nature of the test-organism. Sahni (1966) considered that failure or poor utilization of a sugar may be due to failure or difficulty in the formation of specific carrier system.

There are numerous reports to suggest that fructose and mannose are substantially inferior in value for the lower fungi, especially when short incubation periods are used. Such responses have been reported for Chytridiales, Blastocladales, Leptomitales, and Saprolegniales (Ajello, 1948; Crasemann, 1954; Ingraham and Emerson, 1954; Bhargava 1943; Whiffen, 1945). *Allomyces macrogynous* responds towards fructose and mannose only after a long incubation period, and addition of slight glucose hastens the response (Sistrom and Machlis 1955). Some of the Leptomitales have been reported to use fructose but not mannose. Golueke (1957) reported that *Apodachlya*, *Sapromyces* and *Rhipidium* utilized glucose, fructose and sucrose but not mannose. Gleason (1968 b) found that *Mindeniella* utilized glucose and fructose but not mannose. However, a recently described member of Leptomitales, viz. *Aqualinderella fermentans* (Emerson and Weston, 1967) is able to utilize both fructose and mannose in addition to glucose (Held, 1970). Majority of the higher fungi use fructose and mannose with almost the same efficiency as glucose. Hasijsa (1970 a) recorded better growth of *Alternaria tenuis* and *A. citri* on mannose and galactose and of *Curvularia pallescens* (1970 b) on galactose than on glucose. Some other reports also indicate superiority of mannose or fructose over glucose, as in *Stachybotrys atra* (Jermyn, 1953) and in *Aspergillus flavus* (Grover and Bansal, 1969). On the contrary, fructose was recorded as a poor source of carbon for *Botrytis con-voluta* (Mass and Powelson, 1972).

Utilization of hexoses is speculated to be dependent on an enzyme system which is constitutive in some and inducible in other organisms. *Helminthosporium sativum* exhibited similar adaptability on glucose and galactose (Lilly and Barnett, 1953). Use of longer incubation period is suggested (Tandon 1967) in all such cases, where there is no response during the initial stages. The efficiency of fructose, mannose or galactose possibly depends upon the capacity of the organisms to convert these hexoses into phosphorylated derivatives of glucose capable of entering the main respiratory

pathways. According to Fruton and Simmonds (1953) these four hexose sugars have the following metabolic relationship in yeast.

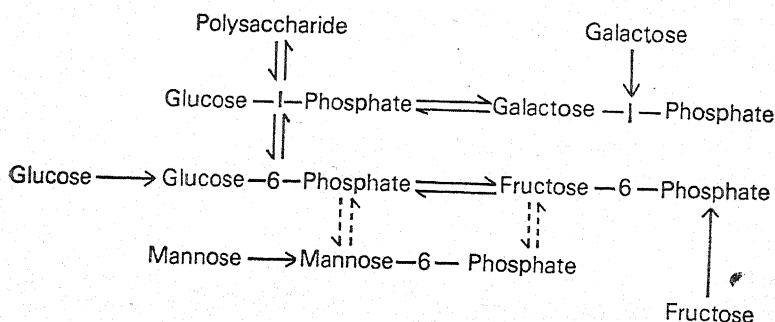


Fig. 4.22. Metabolic interrelationship of hexoses

In case of adaptive growth on fructose, mannose and galactose, the induced enzyme appears to be specific Kinase which brings about phosphorylation of the free sugar. Kita and Peterson (1953) reported the presence of mannose-1-phosphate in the mycelium of *Penicillium chrysogenum* which suggests a pathway of mannose utilization similar to that of galactose in the above scheme. There are possibilities for alternative pathways also but no authentic information is available about them. Several imperfect fungi are known to exhibit variations at the level of species as well as strains for the choice of hexose sugars.

L-sorbose, an epimer of D-fructose with respect to carbon 5 is a poor source for many fungi (Lilly and Barnett, 1953, Bilgrami, 1963; Mehrotra and Kumar 1962 b; Sarbhoy, 1965; Kumar and Grover, 1967; Prasad, 1965; Grover and Bansal, 1969; Hasija and Wolf, 1969; Singh and Tandon, 1967, 70; Manibhushan Rao, 1971), totally unutilizable for some (Crasemann, 1954; Jermyn, 1953; Kumar and Grover, 1967 a) and toxic for others. The toxicity is evidenced by the death of hyphal tips followed by meagre branching of the mycelium below the killed portions (Tatum *et al.*, 1949; Lilly and Barnett, 1953). Lilly and Barnett (1953) recorded inhibitory action of sorbose on growth of many fungi, which increased with increasing concentration of that sugar. The data obtained by them indicated that sorbose in association with maltose or glucose supported good growth of some fungi, but for others it proved to be a poor source. No explanation could however be provided for such varying response. Rizvi and Robertson (1965) found that hyphae of *Neurospora crassa* responded to L-sorbose by spontaneous disintegration of the hyphal

apices, besides the decrease in the colony as well as hyphal diameter. However, gradual addition of dextrose ultimately nullified these effects. Matsushima and Klug (1958) with *Ustilago maydis* tried to obtain a genetical explanation of this problem and found an interaction between genes governing sorbose utilization. The sexually compatible monosporidial lines and their solopathogenic diploid derivative exhibited varying ability to metabolize sorbose. Addition of sorbose delayed the utilization of D-glucose, D-fructose, D-xylose, and L-arabinose by *Curvularia penniseti* (Chandra and Tandon, 1962). Trinci and Collinge (1973) reported that L-sorbose, when added to solid medium, induced profuse branching in *Neurospora crassa* and brought about 90% reduction in their radial growth rate. The hyphae near the colony's periphery exhibited plugging of the septal pores, reduction in the number of vesicles at the hyphal tip as well as autolysis of the mycelium.

Among the pentose sugars, D-xylose, as well as D- and L-arabinose, L-rhamnose and D-ribose have found their application in studies on fungal nutrition. Of these D-xylose is known to support the growth of a large number of fungi and it has even been reported to be superior to glucose for some fungi (Beckman *et al.*, 1953; Perlman, 1948 a; Treschow, 1944). Tandon (1967) reported that D-xylose and L-arabinose were easily utilized by most of the phytopathogenic fungi studied by him and his associates *e.g.* *Curvularia*, *Phyllosticta*, *Botryodiplodia*, *Colletotrichum*, *Gloeosporium*, *Alternaria* and *Pestalotia*. *Cercosporina ricinella* and *Fusarium lini* exhibited poor response to both the pentoses. Some other reports also suggest poor or non-utilization of D-xylose by fungi including *Zygorhynchus californiensis*, *Z-exponens*, and *Z-heterogamons* (Sarbhoy, 1965); *Colletotrichum inamdari* (Hasija, 1965), *Alternaria citri*, *A. tenuis* and *Curvularia pallenscens* (Hasija, 1968, 1970); as well as *Chytriumyces aureus* and *C. hyalinus* (Hasija and Miller, 1971). Cochrane (1958) has sounded a word of caution regarding reports of non-utilization of D-xylose, because autoclaving of the medium results in the transformation of xylose to furfural. Blank and Talley (1941) observed that xylose is better utilized by *Phymatotrichum omnivorum* when the medium is alcohol-sterilized than when it is autoclaved.

Arabinose is utilized by several fungi but as a carbon source its value is generally inferior to both glucose and xylose (Lilly and Barnett, 1956). Moreover, it has been observed that some fungi distinguish between D- and L-enantiomorphs of this pentose and generally it is the L-isomer which is more conducive to fungal growth. *Penicillium urticae* (Ehrensvar, 1955) and *Sporobolomyces salmoni-*

color (Lilly and Barnett, 1956) utilize L-arabinose better than its D-isomer. Poor or non-utilization of arabinose has been reported in various fungi, including *Chytrium aureus* and *C. hyalinus* (Hasija and Miller, 1971), *Pyricularia oryzae* (Manibhushan Rao, 1971), *Alternaria citri* and *A. tenuis* (Hasija, 1968, 1970) *Pestalotia pauciseta* and *Colletotrichum gloeosporioides* (Prasad, 1965), and *Colletotrichum inamdari* (Hasija, 1965). However, in many such studies the isomer used is not specified, which may lead to erroneous conclusions. Steinberg (1942) found that *Aspergillus niger*, though grew well on L-arabinose, it did not grow at all on its D-isomer. Reports of adaptive growth on arabinose after prolonged incubation period are also available. Lilly and Barnett (1953) observed that *Endoconidiophora adiposa* was able to utilize arabinose after longer incubation period in presence of a secondary carbon-source. Similarly, Manibhushan Rao (1971) observed that one of the mutants of *Pyricularia oryzae* was able to grow well on arabinose particularly after 12 days of incubation.

Utilization of D-ribose by fungi has been little investigated, but the available reports suggest it to be a poor source of carbon. Steinberg (1942) did not record any mycelial growth of *Aspergillus niger* on D-ribose, but the same amount of L-ribose in the medium yielded little growth (5 mg in Dry-weight) of the fungus. Hasija and Wolf (1969) also obtained only traces of growth of *A. niger* on D-ribose. Similarly, *Chytrium aureus* and *C. hyalinus* (Hasija and Miller, 1971), do not grow on D-ribose, while a different strain of the latter produces only traces of growth. On the contrary, Tandon and Bhargava (1962) recorded best mycelial growth of *Fusarium solani* on D-ribose; *Botryodiplodia ananasae* and *Macrophomina phaseoli* were also able to utilize D-ribose.

Methylpentoses are derived from hexose sugars by replacement of the primary alcohol group attached to the 6th carbon by a methyl group. Thus, D-isorhamnose, L-rhamnose and L-fucose are derivatives of D-glucose, L-mannose and L-galactose respectively. These sugars have also been little investigated in fungal nutrition. Steinberg (1942) reported that *Aspergillus niger* is able to utilize L-rhamnose but not L-fucose. Other fungi able to utilize L-rhamnose include *Mucor ramannianus* (Margolin, 1942) *Stereum gausapatum* (Herrick, 1940), *Aspergillus oryzae* (Tamiya, 1932), *Botryodiplodia theobromae* (Tandon, 1967), *A. niger* (Hasija and Wolf, 1969) *Pythium aphanidermatum* (Kumar and Grover, 1967 b), *Colletotrichum inamdari* (Hasija, 1965), *Zygorhynchus moelleri* and *Z. heterogamons* (Sarbhoj, 1965). In these studies, the value of L-rhamnose was either similar or in-



ferior or even superior to other pentoses. In a few cases like *Aspergillus flavus* (Grover and Bansal, 1969), *Pestalotia sapotae* and *P. versicolor* (Agarwal and Agnihotri, 1970), *Colletotrichum gloeosporioides* and *Cercosporina ricinella* (Tandon and Chandra, 1962 b), L-rhamnose was better utilized than most of the pentoses and a few hexoses like galactose and or mannose and appeared to be only slightly inferior to glucose and fructose. However, poor response to this sugar is recorded in a number of phytopathogenic fungi including species of *Curvularia*, *Fusarium*, *Cercosporina*, *Phyllosticta*, *Colletotrichum*, *Gloeosporium*, *Alternaria* and *Pestalotia* by Tandon (1967).

Thus, it is apparent that fungi exhibit considerable variation in their utilization of pentoses. Limited experimental data as well as poor methodology employed in earlier investigations lead us to no definite conclusions. Further careful studies are, therefore, imperative.

The 4-carbon and 7-carbon sugars have been practically left untouched, except a few isolated reports. Among tetroses, D-threose has been tested as carbon source by Steinberg (1942) for *Aspergillus niger* and was found to be unutilizable, while erythrose was reported to be utilizable for fungi (Perlman, 1965). The only heptose, studied so far is D-mannoheptulose which was also unsuitable for *A. niger* (Steinberg, 1942). Further studies with naturally occurring tetroses and heptoses may, therefore, be rewarding.

### Sugar Alcohols

Sugar alcohols, which are obtained by reduction of monosaccharides, also vary in their nutritive value. Brock (1951), Grewal (1954), Wolf (1955) and Tandon (1967) have recorded diverse values of these substances for a variety of fungi studied by them. Such diversities are also known within different strains of *Colletotrichum gloeosporioides* (Tandon, 1965). In most of the investigations, hexitols have been employed, and very limited amount of information is available with regard to sugar-alcohols derived from other monosaccharides. Available reports suggest that generally the sugar alcohols are less efficient carbon sources than their corresponding sugars. Among the sugar-alcohols, mannitol appears to be most acceptable to fungi (Tandon and Grewal, 1954; Tandon and Chandra, 1962 b; Sarbhoy, 1965; Singh and Tandon 1967), and in some cases it may be as efficient as glucose (Obaton, 1932; Johnson and Jones, 1941; Cochrane, 1952; Misra and Mukherjee, 1962). However, Perlman (1950), Mehrotra (1951), Fergus (1952) Agarwal (1958) Agarwal and Sinkhede (1959), as well as Ghosh and Tandon (1967) recorded poor or no response of fungi towards mannitol. Bhargava (1945) and



Cantino (1949 a) reported poor utilization of mannitol by some lower Phycomycetes. The other two hexitols, viz. sorbitol and dulcitol, are derivatives of glucose and galactose respectively. Of these sorbitol utilization by fungi seems to be more common. Good to moderate utilization of sorbitol has been reported in *Pythium aphanidermatum* (Kumar and Grover, 1967 b), *Zygorhynchus* spp. and some other Mucorales (Sarbhoy, 1962, 1965), *Colletotrichum papayae* *Gloeosporium* spp. (Ghosh and Tandon, 1967), *Alternaria tenuis* (Singh and Tandon, 1967) and *Phytophthora* spp. (Mehrotra, 1951). However, *Colletotrichum gloeosporioides* (Ghosh and Tandon, 1967), *Helminthosporium rostratum* (Agarwal and Sinkhede, 1959) and *Pythium* spp. (Saksena and Mehrotra, 1949) exhibited a poor response to sorbitol. Dulcitol, on the other hand, could support good growth only in case of *Alternaria tenuis* (Tandon and Grewal, 1954), otherwise in most of the work cited above this sugar alcohol was recorded as a poor carbon source. It has often been observed that the organisms which thrive well on dulcitol are also able to utilize galactose (Tamiya, 1932; Schopfer and Blumer, 1938; Bhargava, 1945).

Among the non-hexose derived sugar alcohols, only two, viz. glycerol and erythritol have found some application in fungal nutrition. They produce varied response among different fungi. Organisms like *Memnoniella echinata* (Perlman, 1948 a) *Pythium* spp. (Saksena and Mehrotra, 1949; Kumar and Grover, 1967 b), *Phytophthora* spp. (Mehrotra, 1951), *Alternaria tenuis* (Tandon and Grewal, 1954), *Zygorhynchus* spp. (Sarbhoy, 1965), and *Lophotrichus ampullus* (Kumar and Grover, 1967 a), make good growth with glycerol as the sole carbon source. However, some versatile fungi, like *Aspergillus niger* and *A. oryzae* utilize glycerol poorly (Steinberg, 1942; Tamiya, 1932). Poor to no growth on this sugar-alcohol has been also recorded in some Mucorales (Sarbhoy, 1962) and *Curvularia penniseti* (Tandon and Chandra, 1961).

Very limited information is available on the utilization of erythritol, which suggest further investigation with diverse genera. Tandon and Grewal (1954) in *Alternaria tenuis*, and Grewal (1957) in some anthracnose fungi reported good utilization of erythritol, while species of *Phytophthora* (Mehrotra, 1951) and *Pythium* (Saksena and Mehrotra, 1949) exhibited only poor response.

Sugar alcohols are supposed to be oxidized and phosphorylated before entering the main respiratory pathways. No enzymic studies have, however, been made and the role of phosphorylated intermediates is only conjectural. Their utilization requires a number of steps, before entering the main cycle. The difference in the capability

of the organisms to oxidize the alcohol and then to phosphorylate them is supposed to influence their capacity to utilize sugar alcohols.

### Sugar Acids

In nature fungi come across various sugar acids, particularly uronic acids which are widely distributed in various complex polysaccharides in various kinds of plant-gums, mucilages and pectin. However, utilization of sugar acids by fungi has been very little investigated and the data pertaining to them are very scanty. The few available reports are mainly regarding the utilization of D-gluconic acid, which suggests that this acid could be a good source of carbon for many fungi, like *Aspergillus niger* (Czapek, 1903), *A. oryzae* (Tamiya, 1932) and others (Cochrane and Conn, 1947; Jermyn, 1953). Steinberg (1942) recorded differences in the response of *Aspergillus niger* towards various forms of D-gluconic acid, like 2-keto-D-gluconic acid, 5-keto-D-gluconic acid and D-gluconic acid. However, gluconic acid is known to be unutilizable for fungi like *Chalara quercina* (Beckman *et. al.* 1953), as well as inhibitory to the fungal growth on account of enhancement of pH of the medium due to removal of the acid.

Utilization of uronic acids has also been investigated with *Aspergillus niger*, and the data suggest that the fungus is able to thrive well on D-glucuronic acid (Steinberg, 1942). Earlier Hofmann (1931) had also reported that *A. niger* utilized D-glucuronic acid and D-galacturonic acid as individual sources of carbon.

Among the glycaric acids, which are produced by the oxidation of both aldehyde and the primary alcohol groups of the sugar molecule, only D-glucaric (saccharic) and mucic acids have been studied. Czapek (1903) found D-glucaric acid as a good carbon source for *Aspergillus niger*, but *A. oryzae* utilized it poorly (Tamiya, 1932). Mucic acid, which is an oxidation product of galactose, is also reported to be well utilized by *A. niger* (Steinberg, 1942).

### Glycosides

Simple glycosides are composed of a sugar moiety and an alcohol or phenol component, the simplest examples being those containing a glucose residue and a methyl group. These methyl glucosides have been investigated for their role in fungal nutrition to a limited extent, and the data suggest that the fungi are generally able to utilize the  $\beta$ -isomers more effectively than their  $\alpha$ -counterparts. Dox and Neidig (1912) found that *Aspergillus niger* completely and rapidly utilized  $\beta$ -methyl glucoside, and its  $\alpha$ -isomer was poorly utilized even after prolonged incubation. However, the latter's utilization was accelerat-

ed in the presence of sucrose (Dox and Roark, 1920). Poor response towards  $\alpha$ -methylglucoside was recorded for *A. oryzae* also (Tamiya, 1932). It has been suggested that such specific response towards isomeric forms of glycosides are probably due to specificity of enzymes as well as due to more frequent occurrence of  $\beta$ -glucosidases among fungi. Also, their ability to utilize different glycosides seems to be related to their ability to consume the component sugar residues. Several members of Saprolegniaceae are able to utilize amygdalin through its hydrolysis into gentiobiose and mandelonitrile (Bhargava, 1943).

### Oligosaccharides

Sugars with two or more monosaccharide units linked together through glycosidic bonds are known as the oligosaccharides. Their nature depends upon their component monosaccharides and the pattern of the glycosidic bonds. Trisaccharides like raffinose and disaccharides like sucrose, maltose, cellobiose, lactose and melibiose have found greater applicability in fungal nutrition. Some of the recent studies on these sugars are supported by simultaneous chromatographic analysis of the media as well as the changes in the pH of the culture solution. Since the utilization of complex carbohydrates by fungi is now known to be accomplished by a set of enzymes known as the transglycosidases, therefore, simultaneous synthesis of transient oligosaccharides *in vivo* and/or *in vitro* is an associated feature during the utilization of oligo- or polysaccharides. The details have been discussed in the next chapter.

Sucrose, which is a major sugar component of photosynthetic plants has generally been reported to be good carbon source for plant pathogenic fungi (Tandon, 1957). Practically all of them are known to convert sucrose into glucose and fructose before utilization. Simultaneous chromatographic analysis of the media (Bilgrami, 1967) has shown that out of the two component monosaccharides, the utilization of glucose fraction is comparatively rapid. Sucrose for a majority of imperfect fungi, is usually as efficient as a synthetic mixture of glucose and fructose, though its value is generally recorded to be inferior to that of glucose as individual carbon source. Chytridiales (Ajello, 1948; Crasemann, 1954) and Mucorales (Satina and Blakeslee, 1928; Raizada, 1957; Sarbhoy, 1965) are either incapable of utilizing sucrose or make very fragmentary growth (Hasiga and Miller, 1971). However, species of *Absidia*, *Actinomucor*, *Chaetocladium* and *Mortierella* were found to grow well on sucrose (Sarbhoy, 1962; 1964). Sucrose is also available to various Leptomi-

tales including *Apodachlya*, *Sapromyces*, *Rhipidium* (Gloueke, 1957); *Mindeniella* (Gleason, 1968 b); and *Aqualinderella fermentans* (Held, 1970). *Sclerotium cepivorum* (Papavizas, 1970) made excellent growth on this sugar.

STB-6B

Maltose, which occurs in nature as a product of starch hydrolysis, has been reported to be used efficiently by a majority of fungi and even those which have limited substrate-ranges, are known to thrive well on it. There are several reports which show a comparative preference for maltose over glucose. Such organisms represent every class of fungi and include *Saprolegnia delica* (Bhargava, 1945), *Aspergillus nidulans* (Mehrotra and Agnihotri, 1961) *Coprinus* sp. (Johnson and Jones, 1941), *Phymatotrichum omnivorum* (Blank and Talley, 1941), *Stachyobotrys atra* (Jermyn, 1953), *Fusarium oxysporum* f. *nicotianae* (Wolf, 1955) and *Pestalotia banksiana* (Tandon and Bilgrami, 1958). This disaccharide is an excellent source for *Sclerotium cepivorum* (Papavizas, 1970). Maltose, before utilization is converted into glucose, and synthesis of transient oligosaccharides in the culture solution is an associated feature of different groups of fungi. Some of the records are tabulated in Table 5.2. The observations distinctly establish that the cleavage of maltose is accomplished by transglycosidases. Partial hydrolysis of transient oligosaccharides yields glucose and maltose in different proportions, whereas on total hydrolysis only glucose is obtained. Few fungi, like *Polychytrium aggregatum* (Ajello, 1948), *Entomophthora* sp. (Wolf, 1951) and *Chytridium* sp. (Crasemann, 1954) are unable to grow on maltose, while certain others like *Penicillium digitatum* (Fergus, 1952), *Diplocarpon rosae* (Shirakawa, 1955) and *Monilinia fruticola* (Lilly and Barnett, 1953) utilized it very poorly. Some members of Leptomitales, like *Apodachlya*, *Sapromyces* (Gloueke, 1957) and *Mindeniella* (Gleason, 1968\*b) failed to utilize maltose, while *Aqualinderella fermentans* utilized this disaccharide well (Held, 1970). Limitation of the necessary enzymes appears to be the probable reason for poor and slow availability of maltose in the above mentioned cases.

Another glucose yielding disaccharide, the cellobiose, where the two glucose units are linked in  $\beta$ -position has found only limited application in fungal nutrition. This disaccharide is produced as an intermediate substance during the utilization of cellulose. A large number of fungi, like *Chytrium aureus* (Hasija and Miller, 1971), *Sclerotium cepivorum* (Papavizas, 1970), *Aqualinderella fermentans* (Held, 1970), *Pyricularia oryzae* (Manibhushan Rao, 1971), *Botryodiplodia theobromae* (Srivastava and Tandon 1969 b) and species of *Colletotrichum* and *Gloeosporium* (Ghosh *et al.*, 1965) show satisfac-

tory response towards it, while several other plant pathogenic fungi (Tandon and Bilgrami 1957, 58) and some lower Phycomycetes (Gloucke, 1957; Gleason, 1968 b) are not able to use it well. The utilization of this sugar is also accomplished by transglycosidases (Bilgrami, 1964, Ghosh *et al.* 1965).

Mushroom sugar trehalose, is also a glucose containing disaccharide and is generally available to the fungi (Cochrane, 1947); Wolf, 1951; Jermyn, 1953; Ghosh *et al.*, 1965; Srivastava and Tandon, 1969; Manibhushan Rao, 1971). Mucorales (Sarbhoy, 1962) show a poor inclination for this substance.

Reaction of fungi towards lactose, a sugar from mammalian milk, is generally very unsatisfactory (Lilly and Barnett, 1953, Ghosh *et al.*, 1965; Bansal and Grover, 1969). Some Mucorales (Sarbhoy, 1962) and Aspergilli (Mehrotra and Agnihotri 1961) as well as some species of *Colletotrichum* (Misra and Mahmood, 1960; Tandon and Chandra, 1962; Hasija, 1965) use this milk-sugar well. Moyer and Coghill (1946) reported that individually lactose was not satisfactory, but even a trace of any good source of carbon considerably improved its response. Synthesis of transient oligosaccharide during the course of its utilization by *Aspergillus oryzae* (Wall-enfels, 1951) and species of *Phyllosticta* (Bilgrami, 1957, 64) is attributed to transgalactosidases.

Melibiose in which glucose and galactose units are linked at  $\beta$ -position, has been used to a very limited extent in fungal nutrition, and is generally a poor source (Tandon and Bilgrami, 1958; Sarbhoy, 1962; Ghosh *et al.* 1955). Good response is however, recorded for *Botryodiplodia theobromae* (Srivastava and Tandon, 1969) and two species of *Colletotrichum* (Ghosh *et al.* 1965). Transglycosidases are operative during its utilization (Tandon and Bilgrami 1957; Srivastava and Tandon, 1969).

Trisaccharide raffinose, is reported to be poor for lower fungi (Mehrotra and Kumar, 1952 a, Sarbhoy, 1954; Hasija and Miller, 1971). For pathogenic Deuteromycetes it is generally good (Tandon and Chandra, 1962; Agarwal and Agnihotri, 1970; Papavizas, 1970; Kapoor and Tandon, 1970) but species of *Phyllosticta* (Bilgrami and Tandon, 1957) and *Pyricularia oryzae* (Manibhushan Rao, 1971) show only a limited response towards this sugar. Enzyme  $\beta$ -fructosidase (invertase) is functional during the earlier stage of its utilization, because fructose and melibiose are easily spotted in the culture-solutions having raffinose as the sole source of carbon. Synthesis of few transient oligosaccharides during its utilization by *Ceratocystis fimbriata* and *Thielaviopsis basicola* (Wilson and Lilly, 1958), two-

species of *Pestalotia* (Tandon and Bilgrami, 1958), *Colletotrichum papayae* (Ghosh *et. al.*, 1965) and *Botryodiplodia theobromae* (Srivastava and Tandon, 1969) is known. Its unsatisfactory utilization is attributed to the poor response of fungi to melibiose (Bilgrami, 1963). Initiation of growth on this substance appears to be totally at the expense of fructose fraction (Tandon, 1967). Considerable diversity is recorded in the response of some species of *Phyllosticta* (Bilgrami, 1965) towards the combination of various components of this trisaccharide. When attacked at  $\alpha$ -linkage raffinose yields galactose and sucrose, but this is generally not common when it is used by filamentous fungi.

### Polysaccharides

In nature, fungi come across a variety of complex polysaccharides, either as structural or nutritional components of plant cells. The structural polysaccharides, like cellulose, pectin chitin etc. are the constituents of cell-walls and act like complex physical and chemical barrier. Commensurate to their function, these structural polysaccharides are generally complex insoluble linear polymers quite suitable for their protective role. The nutritional polysaccharides like starch, glycogen, inulin etc. are generally the forms in which the carbohydrates are stored, and they are branched chain polymers of comparatively better solubility.

Fungi generally utilize both these groups of complex polysaccharides, albeit with varying degrees of efficiency. The general pattern of their utilization involves the initial break-down of these complex polymers into their constituent mono- or disaccharide units, through the agency of various specific enzymes of enzyme systems generally of extracellular origin. However, due to various experimental handicaps, primarily due to the complex nature and little solubility of these substances and secondly due to the indirect approach of most of the earlier workers, their nutritional efficiency *in vitro* have not been evaluated very well.

**Cellulose.** Cellulose is the most abundant natural organic compound and comprises about one-third of all vegetable matters. Being the largest available source of organic carbon, the value of this polysaccharide for heterotrophs in general and the fungi in particular can never be over-emphasized. It has been suggested that the majority of fungi are able to utilize this substance (Normal and Fuller, 1942) and actually the fungi play a major role in its decomposition in nature as compared to other cellulose-decomposing microbial groups. However, our understanding of the complete process involved in the

fungal utilization of cellulose *in vitro* and particularly *in vivo* is far from complete.

A mass of data on cellulose utilization by fungi has come forth as a result of extensive investigations including excellent reviews by Siu (1951) and Venkata Ram (1958). From records, it appears that fungi differ widely in their capacity to utilize this substance. Even different strains of the same fungal species exhibit variations in their cellulose-degrading abilities (White *et al.*, 1948). Also, fungi respond differently to the various cellulosic substrates employed (Tracey, 1953; Venkata Ram, 1956). However, with regard to the rate of utilization, fungi generally show a common trend, and utilize cellulose at a slower rate than its hydrolytic product, the glucose. This has been attributed to the insolubility of cellulose, which limits the activity of the enzyme cellulase to the surface, or to insufficient enzyme-synthesis (Lilly and Barnett, 1951).

A long list of fungi capable of cellulose-degradation, was compiled by Siu (1951), and some recently reported fungi are tabulated in Table 5.1. General distribution of cellulolytic fungi, in different taxonomic groups appears to be such that they are mostly concentrated among Ascomycetes, Basidiomycetes and Deuteromycetes. The few phycomycetous genera reported to be cellulolytic, being to Chytridiales (Whiffen, 1945; Crasemann, 1954), Saprolegniales (Bhargava, 1943; Saksena and Bose 1944 and Mullins, 1973) and the Peronosporales (Mehrotra, 1949), while none of the Blastocladales and Mucorales are known to be cellulolytic. Members of the Homobasidiomycetidae are the most active decomposers of cellulose.

However, all these generalisations have to be interpreted with caution as both interspecific as well as intraspecific variations in cellulolytic activity are known to occur in fungi. Also, many variable factors associated with the experimental conditions, like availability of some major and minor elements and nitrogen, the pH and temperature ranges, presence of some other carbon source, etc. influence the intensity of cellulolytic activity. Explanation for the differences in the activity of the same fungus *in vitro* and *in vivo*, e.g. *Myrothecium verrucaria* and *Memnoniella echinulata* (Siu, 1951) may also be sought in one or the other of these factors.

**Hemicelluloses.** Nutritional efficiency of hemicelluloses for fungi has been little investigated primarily due to their complex and obscure nature as well as the methods of their isolation.

The extracted hemicelluloses generally contain pentosans, hexosans and polyuronides, among which the pentosans in general and the mannans in particular predominate (Whistler and Smart, 1953). The



limited investigations with purified pentosans indicate that most of the fungi employed were able to utilize them (Hawkins, 1951; Treschow, 1944; Jermyn, 1953; Machlis 1953). Degradation of hemicelluloses by fungi *in vivo* i.e. in wood (Robak, 1942; Heuser *et al.* 1949) and other vegetable materials (Schmidt *et al.*, 1923; Norman, 1931) have also been fragmentary. It seems that the fungi are more active in the decomposition of hemicelluloses in the early stages and are followed by bacteria and actinomycetes. Also, species belonging to all major groups of fungi are able to decompose hemicelluloses, and the number of such fungi is far greater than those able to utilize cellulose.

**Chitin.** This linear polymer of N-acetyl, D-glucosamine is a structural component of fungi as well as some higher invertebrates. Although fungi have been found to grow on chitinous materials both in soil (Skinner and Dravis, 1937) and water (Ajello, 1948; Crasemann, 1954) or on the exoskeleton of insects practically no attempt has been made to evaluate the nutritional efficiency of chitin and mode of its utilization by fungi *in vitro*. Reisert and Fuller (1962) studied the production of the enzyme chitinase by the species of phycomycetous fungus *Chytriumyces*. Recently, Reisert (1972) has studied the production and yield of an exocellular chitinase system in *Chytriumyces hyalinus* by using  $^{14}\text{C}$ -labelled chitin. Further studies on this aspect with other fungi are full of possibilities.

**Starch.** Starch is generally found as a storage compound in green plants in the form of insoluble grains. Majority of fungi utilize this polysaccharide with high degree of efficiency (Table 4.1) through amylase activity. A few fungi of diverse taxonomic affinities, however, appear to lack amylase activity and, therefore, they fail to grow on starch as the sole carbon source. This type of correlation has actually been demonstrated in *Penicillium digitatum*, which is unable to utilize starch on account of its inability to synthesize amylase (Holden, 1950). Some other fungal species which fail to grow on starch are as follows:

- |                                     |                               |
|-------------------------------------|-------------------------------|
| 1. <i>Rhizophlyctis rosea</i>       | Quantz (1943).                |
| 2. <i>Entomophthora</i> spp.        | Wolf (1951).                  |
| 3. <i>Penicillium deigitatum</i>    | Fergus (1952).                |
| 4. <i>Chalara quercina</i>          | Beckman <i>et al.</i> (1953). |
| 5. <i>Tricholoma imbricatum</i>     | Norkrans (1950).              |
| 6. <i>Psalliotia bispora</i>        | Treschow (1944).              |
| 7. <i>Ustilago violacea</i>         | Schopfer and Blumer (1938).   |
| 8. <i>Pestalotiopsis versicolor</i> | Agarwal and Ganguli (1960).   |
| 9. <i>Sclerotinia libertiana</i>    | Kakeura (1946).               |



Nutritional efficiency of starch has often been reported to be better than its hydrolytic products *viz.* glucose. This has been attributed to the presence of some growth factors in the form of impurities, or may be that the slower hydrolysis of starch causes lesser accumulation of acids. Starch utilization has also been correlated with its partial hydrolysis product maltose, and organisms thriving well on starch generally have been found to exhibit good response to maltose also. Crasemann (1954), however, found that a *Chytridium* spp. responded to starch fairly well but could not utilize maltose. Similarly, *Phymatotrichum omnivorum* fails to grow on starch granules unless autoclaved (Blank and Talley 1941). Such observations have clearly exposed that much more remains to be understood regarding the utilization of starch by fungi particularly *in vivo*.

Dextrin, or the modified, starch has also been considered as a good source of carbon, but further investigations are needed to explain their often repeated superiority over starch. Beckman *et al.* (1953) reported that *Chalara quercina* although unable to utilize starch, grew well on dextrin. Tamiya (1932) found that *Aspergillus oryzae* attained better growth on dextrin than on starch.

Glycogen, the primary storage polysaccharide in fungi (Zalokar, 1965) has been little investigated in fungal nutrition. The available reports suggest that it is generally a good source of carbon. Glycogen resembles the amylopectin component of starch and hence it is presumed that this polysaccharide may be unutilizable by fungi with inability to use starch. Glycogen is well distributed in the fungal hyphae and reproductive bodies.

Inulin, a D-fructose rich polysaccharide, is believed to consist of a sucrose unit linked to a large number of fructose residues. Nutritional value of inulin for fungi generally appears to be good, although not universal. Fungi utilize this polysaccharide through enzymatic degradation, and the enzyme inulase is known from very early investigations (Bourquelot, 1893 b). This polysaccharide is not attacked either by amylase or sucrase (Cochrane, 1958), but interestingly inulase is active on sucrose. Hence Nakatsu (1956) suggested that inulase may be a transfructosidase. Obviously, further study is needed to reveal the true nature of this enzyme, and its mode of action. Some recent investigations on inulin utilization by *Aspergillus* spp. (Agnihotri, 1963), and *Colletotrichum* and *Gloeosporium* spp. (Ghosh and Tandon, 1965) have also indicated that this polysaccharide is hydrolyzed during utilization. However, chromatography of the culture solution revealed only fructose and no glucose. This has been attributed to slow utilization and low glucose-content of inulin. In

both these studies, transient oligosaccharides were also detected.

TABLE 4.1

SHOWING UTILIZATION OF INULIN, DEXTRIN AND STARCH  
BY SOME FUNGI

	Inulin	Author's	Dextrin	Starch
1. <i>Cercosporina ricinella</i>	Good	Tandon & Chandra (1962)	Poor	Good
2. <i>Colletotrichum gloeosporioides</i>	Good	Tandon & Chandra (1962 b)	—	—
3. <i>Curvularia</i> spp.	—	Singh & Tandon (1971)	Good	Good
4. <i>Alternaria tenuis</i>	Good	Singh & Tandon (1967)	—	Good
5. <i>Colletotrichum</i> spp. and <i>Gloeosporium</i> spp.	Good	Ghosh & Tandon (1965)	Good	Good
6. <i>Pestalotia pauciseta</i>	Good	Prasad (1967)	Good	—
7. <i>Botryodiplodia theobromae</i> <i>C. gloeosporioides</i>	Poor	Do	Poor	—
8. <i>Curvularia pallescens</i>	—	Hasija (1970)	—	Good
9. <i>Pestalotiopsis versicolor</i>	—	Agarwal & Ganguli (1960)	—	Poor
10. <i>Pestalotiopsis</i> spp.	Good	Dube (1966), Srivastava (1965)	Good	Good
11. <i>Helminthosporium rostratum</i>	—	Agarwal & Sinkhede (1959)	—	Moderate
12. <i>Curvularia penniseti</i>	—	Agarwal (1958)	—	Good

### Organic Acids

Non-fatty organic acids like citrate, succinate and malate, which are the usual products of glucose metabolism are more acceptable to fungi than others, though in general the organic acids are poor carbon sources. It has been suggested that because of low pH of the media containing organic acids, the cells are often impermeable. It is also on record that utilization of neutralized organic acids causes the rise in pH of the culture medium, which often interferes with the growth. These observations find support from the fact that organic acids are utilized by fungi, if nitrogen is supplied in the form of ammonium salts (Leonian and Lilly, 1940; Reischer, 1951; HacsKaylo *et al.* 1954; Srivastava 1955). Certain organic salts like citrate and tartarate are known to cause chelation of inorganic ions. The good

TABLE 4.2

## UTILIZATION OF ORGANIC ACIDS BY SOME FUNGI

Organisms	Source	Value	Author's
1. <i>Absidia orchidis</i> , <i>A. ramosa</i> , <i>Actinomucor elegans</i> , <i>Cunninghamella bertholletiae</i> , <i>Chaetocladium hesseltinii</i> , <i>Zygorhynchus</i> spp. <i>Mortierella indica</i>	Malic acid Tartaric acid	Poor or no growth	Sarbhoy (1962, 65)
2. <i>Allomyces javanicus</i>	Acetate	Good	Machlis (1953)
3. <i>Pythium aphanidermatum</i>	Citric acid	Good	Knmar & Grover (1967)
4. <i>Pythium aphanidermatum</i>	Acetic acid, Oxalic acid	No growth	Kumar & Grover (1967)
5. <i>Leptomitus lacteus</i>	Lower organic acids	Good	Schade (1940).
6. <i>Aspergillus versicolor</i>	Tartarate	Poor	Barton (1953)
7. <i>A. oryzae</i>	Oxalic acid	Good	Muller (1950)
8. <i>Penicillium janczewskii</i>	Succinate	Good	Brian <i>et al.</i> (1946)
9. <i>P. javanicum</i>	„	Poor	Lockwood <i>et al.</i> (1934)
10. <i>P. digitatum</i>	„	No growth	Fergus (1952)
11. <i>Cercosporina ricinella</i>	Tartaric acid malic acid	Poor	Tandon & Chandra (1962)
12. <i>Memmoniella echinulata</i>	Tartaric acid	Good	Perlman (1948 a)
13. <i>Curvularia penniseti</i>	Tartaric acid	No growth	Tandon & Chandra (1961)
14. <i>Alternaria tenuis</i>	Oxalic and Tartaric acids	No growth	Singh and Tandon. (1966)
15. <i>Colletotrichum gloeosporioides</i> and <i>C. dematium</i>	Tartaric, oxalic, malic, citric acids	Poor	Misra & Mahmood (1960) Chandra (1961) Tandon & Verma (1962)
16. <i>Pestalotiopsis versicolor</i>	Oxalic acid	No growth	Agarwal and Ganguli (1960)
17. <i>Pestalotiopsis versicolor</i>	Tartaric acid	Poor	Agarwal & Ganguli (1960)
18. <i>Pestalotia</i> spp.	Citric, malic, oxalic and tartaric acids	Poor to no growth	Tandon & Bilgrami (1958) Dube (1971 a)
19. <i>Phyllosticta cycadina</i>	Do	Poor	Tandon & Bilgrami (1956)
20. <i>Psalliota bispora</i>	Oxalic acid	Good	Treschow (1944)

or poor effect of chelation, however, depends on the solubility of chelate complexes as well as the metallic salts at the selected culture-pH. The value of some of the non-fatty acids for different fungi is tabulated in Table 4.2 and it appears that their utilization is also very specific.

The monocarboxylic acids, which are also called fatty-acids (because they form fat on esterification with glycerol) are generally unutilizable and toxic to fungi. Some of the fungi like *Leptomitius lacteus* (Schade, 1940) can, however, grow on these compounds as sole carbon source, and are able to utilize even those acids which are definitely toxic to higher forms. Similarly, some of the fatty acids, like acetate support atleast moderate growth of several fungi. For *Allomyces javanicus* (Machlis 1953), acetate is almost equivalent to glucose as a carbon source. Acetate supported the best vegetative growth of all other carbon sources employed for *Lophotrichus ampullus*, a coprophilous ascomycete (Kumar and Grover, 1967 a), although the fungus did not sporulate at all. *Pythium aphanidermatum* (Kumar and Grover, 1967 b) however, did not grow on acetate. Utilization of acetate has also been reported for *Penicillium chrysogenum* (Jarvis and Johnson, 1947) and uredospores of wheat stem-rust (Suryanarayanan and McConnell, 1964). Formic acid is not of much value as carbon source, and supports only poor growth of some fungi (Baba, 1941; Harrold and Fling, 1952). Propionic acid, which is generally used as a fungistatic agent is generally toxic to fungi. However, Reisener *et al.*, (1963), by using labelled propionate showed that uredospores of wheat stem rust utilized carbon 2 and 3 as carboxyl, while carbon 1 was lost as CO<sub>2</sub>. Suryanarayanan and McConnell (1966) studied the uptake and metabolism of propionic acid by uredospores of wheat stem rust. Higher fatty acids, except valeric acid (Reisener *et al.*, 1961), have found little application in fungal nutrition.

### Amino Acids

Many fungi may use amino acids both as a source of nitrogen as well as the sole source of carbon (Nickerson and Mohan, 1953). Likewise, peptone may serve as a carbon source also, besides furnishing nitrogen to the organism. Steinberg (1942 a) studied the utilization of amino acids by *Aspergillus niger* and found that certain "primary" amino acids may in combination be about 1/3rd as efficient as sucrose. Gottlieb (1946) studied the carbon values of individual amino acids for *Penicillium roqueforti* and *Fusarium oxysporum* var. *lycopersici*, and found that their utilization differed.

Glycine and valine were utilized well by *F. oxysporum lycopersici*, while for *P. roqueforti* they were poor carbon sources. Six-carbon straight chain amino acids norleucine and lysine as well as the sulphur containing amino acids cysteine and methionine, however, were not utilizable as carbon-source. Naturally occurring sulphur containing amino acids were also unutilizable by *Alternaria solani*, *Helminthosporium sativum*, *Rhizoctonia solani*, *Fusarium moniliforme*, *Chaetomium globosum*, and *Aspergillus niger*. During utilization of amino acids or peptone, the fungi cause their deamination and release ammonia. This is supposed to be the reason for the rise in pH of the amino acid media due to fungal growth.

## CARBON METABOLISM—I

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**METABOLISM OF COMPLEX CARBOHYDRATES AND DERIVATIVES**

Metabolism denotes the organised chemical activities of the cell, whereby nutrients are transformed into cellular material, waste products and the all important energy. All such metabolic activities of the cell are directed either towards assimilation of complex organic compounds synthesized from simpler basic materials, or towards degradation of complex substances into simpler break-down products. However, the two types of activities are so intimately inter-linked, that the assimilatory and dissimilatory reactions of the cell can hardly be earmarked. The close affinities of these two metabolic aspects are all the more conspicuous in the chemotrophic organisms like fungi, where the entire cellular activity is dependent upon the chemical energy, which in itself is a product of the catabolic functions of the cell.

As has been mentioned in the preceding chapter, the carbohydrates and their derivatives serve as the principal substrate for carbon metabolism in fungi, and in fact perform a dual function, *viz.* (i) they are oxidised as the principal source of chemical energy which is made available to the cell in the form of ATP and reduced phosphopyridine nucleotides; and (ii) they provide most of the carbon required for the assimilation of fungal carbohydrates, lipids, amino acids, enzymes and many other organic cell constituents.

The carbohydrates are assimilated through one or the other glycolytic pathways, which results in the formation of pyruvic acid. The pyruvic acid may undergo further reactions under anaerobic conditions to produce acetaldehyde, ethanol and carbon dioxide, or it may get reduced to lactic acid. Under aerobic conditions, on the other hand, the pyruvic acid participates in cyclic chain reactions of Krebs tricarboxylic acid cycle. Several organic acids including  $\alpha$ -Oxo-

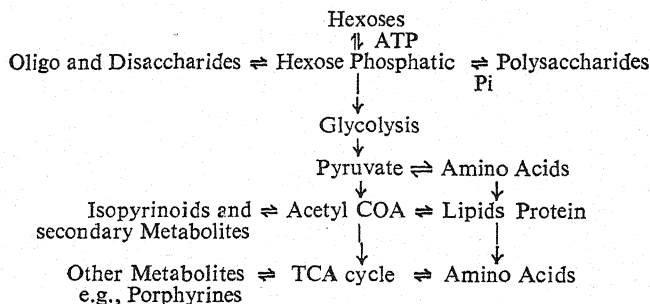
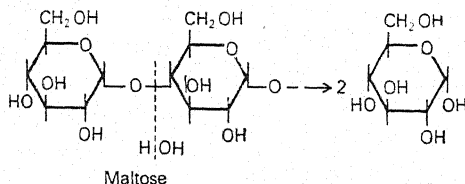


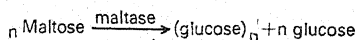
Fig. 5.1. Main steps in carbon metabolism with off-shoots.

glutaric and oxalacetic acids are formed due to a series of enzymatic reactions. These organic acids through transaminase action get converted to essential amino acids like glutamic and aspartic acids. By

1. Hydrolysis of a glucosidic bond



2. Transglucosidation



3. Phosphorylation

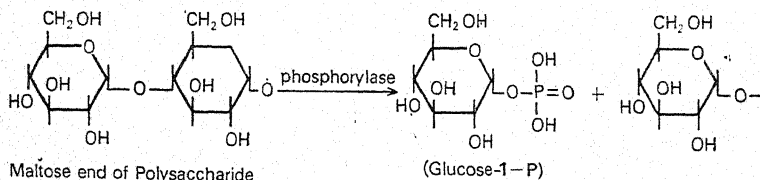


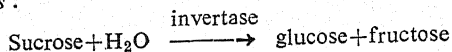
Fig. 5.2. Degradation of complex carbohydrates.

another series of reactions, these amino acids polymerize to form proteins. Polymerization of sugars gives rise to various carbohydrates such as cellulose, starch and chitin. Therefore, during carbon metabolism, it is essentially a change from carbon to carbon as fungi first degrade the complex carbon compounds to simpler ones and then resynthesize them into complex cell-constituents.

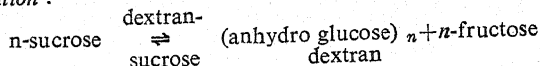
Bulk of the naturally occurring carbohydrates are either poly-

saccharides or oligosaccharides which form most of the reserve as well as the structural materials of plant-body. Under natural conditions, therefore, the fungi largely depend upon the complex carbohydrates for their carbon nutrition. In most of the cases, the complex carbohydrates are first degraded into monosaccharide moieties with the help of extracellular enzymes, before these high molecular-weight compounds can be absorbed by the fungi for their assimilation. The initial degradation of the poly- and oligo-saccharides thus involves the breaking down of glycosidic linkages. Most of the fungi accomplish this feat with the help of specific enzymes or enzyme system through either of the following three types of reactions.

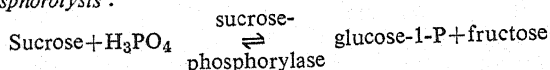
(a) *Hydrolysis* :



(b) *Transglycosidation* :



(c) *Phosphorolysis* :



The reversible nature of the last two reactions (phosphorolysis and transglycosidation) under the influence of the same kind of enzyme amply demonstrates the close affinities between the dissimilative and assimilative reactions of the cell.

## POLYSACCHARIDES

### (A) DEGRADATION OF POLYSACCHARIDES

In nature, fungi come across various types of polysaccharides and their derivatives in different kinds of mixtures. With an effective battery of constitutive as well as inducible enzymes the fungi are able to utilize most of the complex compounds available in their environment. Fungal degradation of many of the naturally occurring polysaccharides has been traced to an appreciable extent, particularly during the last two decades.

#### (1) Degradation of Cellulose

Cellulose decomposition is known to be accomplished by a variety of living beings, including fungi, bacteria, actinomycetes, protozoa, and some insects. However, fungi are considered to be more active cellulose decomposers, and except for some lower fungi most of the members belonging to Ascomycetes, Basidiomycetes as well as Fungi



Imperfecti are able to degrade this polysaccharide. Much of the information regarding the enzymatic break-down of cellulose have been gathered from studies on soluble cellulose (cf. native cellulose) and with wood-decaying fungi. Moreover, in many of the earlier studies, usually crude techniques and impure preparations of cellulose were employed. However, enzymatic degradation of this major constituent of plant cell-wall does occur in nature. It is also well established that cellulases are extracellularly produced by many fungi. Norkrans (1950 b) found that only those cells of *Tricholoma fumosum* produced this enzyme, which were in contact with cellulose or lichenin (resembles cellulose but abounds in 1, 3-glycosidic bonds) and that the enzyme was produced extracellularly in cultures. Further studies have also indicated that the cellulases are produced abundantly in cultures with cellulose as the sole source of carbon. Therefore, most of the cellulase enzymes are supposed to be inducible. However, production of cellulases in the absence of cellulosic substrates has also been reported by Strider and Winstead (1961) in *Cladosporium cucumerinum* and by Winstead and McCombs (1961) in *Pythium aphanidermatum*. Such reports indicate their constitutive nature also. Cellulolytic activity of various other fungi *in vitro* and/or *in vivo* have been tabulated in Table 5.1.

Studies on cellulose degradation *in vivo* by some phytopathogenic fungi, have shown that cellulases are often produced to break the host barrier for getting an entry into the host tissue. Bateman (1963, 64) in case of *Alternaria solani* as well as Hancock and Miller (1965 a) in *Colletotrichum trifolii* found strong cellulolytic activities preceding the hyphal penetration of the host cell-wall by the pathogens. Cellulase production by *Colletotrichum lindemuthianum* grown on isolated cell walls, is preceded by formation of pectinase,  $\alpha$ -arabinosidase and  $\beta$ -xylosidase and is followed by  $\beta$ -glucosidase and  $\alpha$ -galactosidase (English *et al.* 1971). *Fusarium oxysporum* and *Verticillium albo-atrum* are able to cause wilt symptoms in their hosts due to plugging of their vascular elements by the macromolecular products of enzymatic degradation of cellulose by these fungi. Studies on *Fusarium* wilt by Husain and Dimond (1960) as well as by Deese and Stahmann (1962) have also led to such conclusions. According to Norkrans (1963) cellulolytic enzymes seem to be a pre-requisite in certain wilt-diseases. Bateman and Miller (1966), however, are of the view that cellulases do not play any significant role in the maceration of plant tissues. Wood (1960) reported that cellulase may not appear to be very important in the early stages of disease development, but in the later stages of soft-rot, cellulose of

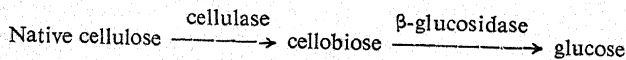
the microfibrils is definitely attacked by the soft-rot pathogens.

TABLE 5.1  
SHOWING SOME PHYTOPATHOGENIC FUNGI PRODUCING  
CELLULASES

Fungi	References
<i>Alternaria tenuis</i>	Tandon & Srivastava (1949)
<i>Alternaria</i> spp.	Van Parijs (1961)
<i>Botrytis allii</i> , <i>B. squamosa</i> <i>B. cinerea</i>	Hancock <i>et al.</i> (1964 a, b)
<i>Botryosphaeria ribis</i>	Husain & Dimond (1958 a)
<i>Cercospora sorghi</i>	Vidhyasekaran <i>et al.</i> (1971)
<i>Cladosporium cucumerinum</i>	Strider & Winstead (1961), Husain & Rich (1958)
<i>Cochliobolus miyabeanus</i>	Akai (1951)
<i>Colletotrichum lagenarium</i>	Winstead & McCombs (1963)
<i>C. linicola</i>	Klug & Threinen (1957) Van Parijs (1961)
<i>C. phomoides</i>	Schmittthener (1960)
<i>C. trifolii</i>	Hancock & Miller (1965 a)
<i>Fusarium</i> spp.	Venkata Ram (1957, 1959)
<i>F. oxysporum f. lycopersici</i>	Husain & Dimond (1960)
<i>F. roseum f. cerealis</i>	Phillips (1962)
<i>F. solani</i>	Etchells <i>et al.</i> (1958)
<i>F. moniliforme</i>	Foley (1959)
<i>Glomerella cingulata</i>	Husain & Dimond (1958 a), Mohanty & Ada, (1971)
<i>G. tucumanensis</i>	Singh & Hussain (1963)
<i>Helminthosporium</i> spp.	Gilligan & Reese (1954)
<i>Helminthosporium turcicum</i>	Vidhyasekaran <i>et al.</i> (1971)
<i>H. oryzae</i>	Shigeyasu (1951)
<i>Pezizula alba</i> , and <i>P. malicorticis</i>	Edney (1964)
<i>Physalospora obtusa</i>	Husain & Dimond (1958 a)
<i>Phytophthora cactorum</i> , <i>P. citricola</i> and <i>P. parasitica</i>	Mehrotra (1949)
<i>Puccinia purpurea</i>	Vidhyasekaran <i>et al.</i> (1971)
<i>Pyricularia oryzae</i>	Manibhushan Rao (1971)
<i>Pythium aphanidermatum</i>	Winstead & McCombs (1961)
<i>Pythium</i> spp.	Saksena & Jafri (1950)
<i>Rhizopus stolonifer</i>	Srivastava <i>et al.</i> (1959), Spalding (1963)
<i>Rhizoctonia solani</i>	Kohlmeyer (1956) Barker & Walker (1962), Garrett (1962), Bateman (1963 b)
<i>Sclerotium rolfsii</i>	Husain (1958), Bateman (1969)
<i>Sclerospora sorghi</i>	Vidhyasekaran <i>et al.</i> (1971)
<i>Stemphylium botryosum</i>	Hancock & Millar (1965 a)
<i>Stereum sanguinolentum</i>	Bucht & Eriksson (1969)
<i>Verticillium albo-atrum</i>	Whitney <i>et al.</i> (1969)

Besides their role in plant diseases, some sort of relationship between cellulase production and lateral branch formation in *Achlya*, *Saprolegnia*, and *Dictyuchus* has also been observed (Mullins, 1973). Earlier, Fergus (1969) found cellulolytic activity more common in thermophilic fungi than in thermophilic actinomycetes.

The details of the mechanism involved in the break-down of cellulose as well as the exact nature of the cellulase enzyme are yet to be fully understood. That the break down of this polysaccharide involves more than one enzyme has been indicated by early investigators. Pringsheim's (1912) classical concept on this aspect envisages the involvement of at least two enzymes: (i) The cellulase, which is able to split the cellulose chain into cellobiose residues, and (ii) a cellobiase ( $\beta$ -glucosidase) which acts upon the disaccharide and transforms it into glucose residues. However, subsequent investigations have led to varying conclusions, and although extensive reviews on this subject have appeared in the last two decades (Siu, 1951; Siu and Reese, 1953; Gascoigne and Gascoigne, 1960; Reese, 1963, Wood, 1967), further work on this line is necessary for formulation of any general theory. Interestingly two contradictory theories based on studies with the same organism, viz., *Myrothecium verrucaria* were proposed. Whitaker (1953) and Whitaker *et al.* (1954) were able to isolate and purify by electrophoresis a single enzyme (mol. wt. 63,000) which could hydrolyze cellulose to glucose. They concluded that although cellobiose may be formed during the process, it does not necessarily act as an intermediate. Aitken *et al.* (1956), are also of the opinion that a single enzyme converts cellulose to cellobiose, but they consider that a cellobiase is necessary for the production of glucose. There is ample evidence that some wood-rotting fungi including *Collybia velutipes* and *Polyporus annosus* require a  $\beta$ -glucosidase in addition to cellulase to degrade cellulose to glucose (Norkrans, 1957 a, b). The scheme possibly operating in these fungi is as follows:



In sharp contrast to the above findings, Reese, Gilligan and Norkans (1952), Reese and Levinson (1952), Gilligan and Reese (1955) Miller and Blum (1956) and Halliwell (1957) considered at least three enzymes essential for the hydrolysis of cellulose. Later, Norkrans (1963) Petterson *et al.* (1963), Cowling (1965) as well as Petterson and Porath (1965) have suggested that the cellulases may actually comprise a number of chain splitting enzymes, which cause the frag-

mentation of the cellulose chain into the dimer cellobiose and other higher oligosaccharides, like cellotriose, cellotetrose, etc. Petterson *et al.* (1963) found that the fungus *Polyporus versicolor* produced a number of cellulases which differed markedly from each other. Two such cellulases differed in their molecular weight. The one with low molecular weight, *i.e.*, 11,000 attacked the glucosidic bonds, while the other with higher molecular weight (51,000) brought about the hydrolysis of the dismantled chain fragments, *i.e.*, the oligosaccharides. However, the apparent plurality of the cellulases have been ascribed by Thomas and Whitaker (1958) to misinterpretation of electrophoretic data. According to them, there is a single cellulase enzyme, but it forms complexes with different polysaccharides which move at various speeds on electrophorogram and are misjudged. Despite this suggestion the work of Reese and his associates has received wide support in view of their carefully performed experiments. They have concluded that cellulases comprise a number of enzymes, and have proposed the following scheme for the degradation of cellulose:

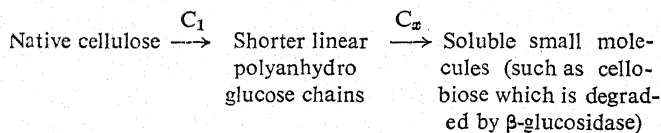


Fig. 5.3. Stepwise degradation of native cellulose  
after Reese *et al.* (1950).

Of these, the distribution of  $C_x$  enzymes was found to be widespread among the micro-organisms, including the non-cellulolytic organisms. On the contrary, the  $C_1$  enzyme is much less common and is restricted only to cellulolytic organisms, which are thus able to degrade the native cellulose. King (1961) has suggested that the  $C_1$  enzyme renders the cellulose fibres vulnerable for  $C_x$  degradation. Similar activity by an enzyme designated as 'S-factor' was noted by Marsh *et al.* (1953), which they found as distinct from  $C_x$  enzyme, although its relation with  $C_1$  enzyme is not yet clear. Recently Bucht and Eriksson (1969) have studied the extracellular cellulase enzyme system of the rot-fungus *Stereum sanguinolentum* and have separated the cellobiase activity from  $\beta$ -glucosidase activity.

Degradation of cellulose microfibrils *in vivo* is little known, although Cowling (1965) has enlisted some structural characteristics of cellulose, which make them accessible to enzymatic break-down: (i) hydration of the fibres, (ii) the degree of crystallinity of cellulose, (iii) the polymerization of the cellulose molecules, (iv) the substances

associated with cellulose, e.g. hemicelluloses, pectin, lignin, protein etc. and the type of linkage with them, and (v) the size and diffusibility of the enzyme molecules in respect to the size and surface properties of the capillaries in between the cellulose microfibrils. All these factors will affect the enzyme action because the enzyme molecules must get into direct contact with the hydroxyl groups of the cellulose-chain before the hydrolysis of this polysaccharide can take place.

Several factors are known to influence cellulase-production by fungi. Stranks (1973) reported that phenethyl alcohol (PEA) and some other specific solvents enhanced cellulase production by several fungi (*Myrothecium verrucaria*, *Trichoderma viride*, *Polyporus versicolor*, and *Lenzites trabea*), and since cellulase synthesis is thought to occur on or within the cytoplasmic membrane, it is suggested that the phenethyl alcohol exerts its influence through causing permeability and solute transport changes. Alternatively, PEA might cause increased release of cellulase from the cell-wall.

## (2) Degradation of Hemicelluloses and Pentosans

Comparatively, very little is known about the enzymatic breakdown of hemicelluloses, obviously on account of their not too well known chemical structure, the difficulties in their extraction and their sparing solubility. Informations regarding hemicellulytic enzymes have mostly come from studies with wood destroying fungi and bacteria, besides a few members of Saprolegniaceae (Bhargava, 1943; Saksena and Bose, 1944) and *Phytophthora* (Mehrotra, 1949). O'Dwyer (1939, 40) found that *Aspergillus oryzae* attacked the crude hemicellulose fraction obtained from wood and produced free sugars and a soluble polysaccharide. Among the hemicelluloses certain pentosans have received the maximum attention particularly the xylans, which are the most abundant of all pentosans. Some crystalline xylanases have actually been obtained from culture filtrates of quite a few fungi. Grassmann *et al.*, (1933) in *Aspergillus oryzae* and Hawkins (1915) in *Glomerella cingulata* found xylanase activities. Sorensen (1952, 53) found that *Chaetomium globosum* produced appreciable amounts of xylanase in cultures containing xylans. He distinguished two fractions of this enzyme, one was extracellular, which degraded xylans to xylobiose and the other was an intracellular component which hydrolyzed the xylobiose. Strobel (1963) has described a xylanase from grapes infected with the fungus *Diplodia viticola*, which was able to degrade the xylans from grapes, yielding xylose, arabinose, glucose, xylobiose, xylotriose, xylotetrose and

xylopentose. Two distinctly different kinds of xylanase activities have so far been noticed. Some xylanases are able to cause end-cleavage in xylan chains and produce xyloses, while others attack the chain in random fashion to produce short chain fragments, which are subsequently degraded. The limited amount of literature on hemicellulases have been reviewed by Pigman (1951), and Gascoigne and Gascoigne (1960).

Some fragmentary investigations on a few other pentosans have also been made but are in no way helpful in the understanding of the hemicellulases and their mechanism of action. Ehrlich and Kosmahly (1929) reported that a crude enzymatic preparation from *Aspergillus oryzae* hydrolyzed arabans, a pentosan made up largely of L-arabinose. Ratajak and Owens (1942) observed that a pure arabogalactan was degraded by an enzyme obtained from *Aspergillus niger*. Sorensen (1952) reported enzymatic break-down of yet another pentosan, viz., mannans, by an enzyme produced by *Chaetomium globosum*. Interestingly, certain well purified fungal cellulases also act upon xylans (Bishop and Whitaker, 1955; Bishop, 1956), and these enzymic preparations exhibit the two activities quite distinctly. A xylanase enzyme was detected in the stem-lesions of alfalfa caused by *Colletotrichum trifolii* (Hancock and Miller, 1965 a). All such work can hardly be counted for anything more than a descriptive value, and much more investigations are needed on this aspect.

### (3) Degradation of Starch

Natural starches comprise two separable compounds the amylose with an unbranched chain structure and the amylopectin possessing a branched chain structure. The branches also consist of glucose units but they are joined by  $\alpha$ -1, 6, glucosidic linkages. Each glucose residue at a branch point thus forms three glucosidic bonds, one each at carbon 1, 4 and 6. Although both these components of starch are hydrolyzed by the enzyme amylase (diastase), the products of their degradation differ, mainly on account of their structural differences. Also, the following two kinds of amylases have been identified (Myrback and Neumuller, 1950), which differ in their mode of action and therefore, produce different degradation products:

(i) *Alpha-amylases*, which are also called endoamylase, dextrinizing amylase etc., are distributed in animals (Pancreatic or salivary amylase) and microorganisms including fungi. These amylases attack the  $\alpha$ -1, 4, glucosidic bonds of both amylose and amylopectin (but not of the disaccharide maltose) in a random manner and produce dextrin and maltose, which by the action of a glucogenic enzyme

system, produce glucose.

(ii) *Beta-amylases*, which are also designated as exoamylase, saccharifying amylase etc. are found in seeds, such as barley malt. These  $\beta$ -amylases are able to split the polysaccharide chain in a successive manner, removing the disaccharide maltose units from the non-reducing ends of the chain.

Amylases are considered to be widely distributed among fungi, and cell-free culture fluids of many fungi have been found to hydrolyze starches and their related compounds the glycogens. Holden (1950 b), however, reported that *Penicillium digitatum* did not form any detectable amylase in culture and concluded that the fungus might be deficient for that enzyme. The present evidences indicate that the amylases produced by fungi in general belong to the group  $\alpha$ -amylase. Leopold and Starbanow (1943), Lemense *et al.* (1947), as well as Meeuse (1952) did not find any indication of the occurrence of  $\beta$ -amylases among fungi. The saccharification of starch appears to be principally due to an  $\alpha$ -amylase and a glucogenic enzyme system. Little or no  $\beta$ -amylase activity has been observed in some molds. The gluc amylase enzyme of *Rhizopus delemar* (Phillips and Caldwell, 1951 a and b) which is also referred as amyloglucosidase or gamma-amylase, resembled the  $\beta$ -amylase in a few respects only, and differed from the  $\alpha$ -amylase appreciably. It has rather been confirmed by repeated findings, that the purified enzymatic product of *Aspergillus oryzae* comprise an alpha-amylase (Caldwell *et al.* 1945. Fischer and de Montmillon, 1951 a; Underkoffler and Roy, 1951), although in crude amylase preparation from this fungus more than one components were indicated by electrophoresis, as has been reported by Gillespie and Woods (1953). Informative discussions on amylases have been given by Redfern (1950), Caldwell and Adams (1951), Schwimmer (1951), and Adams (1953).

(a) *Hydrolysis of amylose*. The  $\alpha$ -amylase degrades the amylose fraction of starch by random cleavage of the 1, 4,  $\alpha$ -glucosidic bonds and gives rise to a mixture of dextrin and maltose residues. Later a glucogenic enzyme system acting on dextrin and maltose produces glucose. However, pure maltose is produced more or less in quantitative yield, if the amylose is degraded by a  $\beta$ -amylase enzyme, obviously because a  $\beta$ -amylase successively removes disaccharide maltose units from the amylose chain. Maltose, when acted upon by maltase gives rise to glucose residues.

(b) *Hydrolysis of amylopectin*. Similar random cleavage of the  $\alpha$ -1, 4, bonds of amylopectin takes place under the influence of  $\alpha$ -amylase. Since this enzyme is inactive against 1, 6,  $\alpha$  bonds, the ul-



itimate product of amylopectin degradation by  $\alpha$ -amylase, consists of branched and unbranched oligosaccharides, in which the 1, 6'  $\alpha$ -linkages abound. It has been observed that moulds used for saccharifying starches contain  $\alpha$ -amylase, a maltase (gluc amylase or  $\gamma$ -amylase) and a limit dextrinase. These enzymes are produced in different proportions by different molds. When the amylopectin is attacked by a  $\beta$ -amylase, the disaccharide maltose is produced successively from the non-reducing end of the polysaccharide chain, which continues until a branch point is approached. At this point, the enzyme is not able to break the 1, 6'  $\alpha$ -linkage and any further hydrolysis of the chain is checked. These small chain fragments of dextrin are called limit dextrans because their production imposes a limit to further degradation of the polysaccharide by this enzyme.

Glycogens, which resemble much the amylopectin fraction of starch and possess a branched chain structure, are hydrolysed by  $\beta$ -amylase in identical manner to give rise to maltose and limit dextrin.

#### (4) Degradation of Pectic Substances

Investigations into the enzymatic break-down of pectic substances were given a good start by de Bary as early as in the year 1886. Ward (1888) was another contemporary worker in this field. These early investigators made an interesting observation that crude enzymatic preparation from some phytopathogenic fungi destroyed the middle lamella or the cementing substances in between the cells, and thus could macerate the plant tissue. Since then, much ground has been covered in this direction and to-day enzymatic break-down of pectic substances are perhaps the best understood of all the polysaccharides associated with plant cell-wall.

Pectic substances comprise atleast three kinds of compounds, viz., (i) pectic acid, (ii) pectinic acid, and (iii) protopectin. All the three substances are polymers of galacturonic acid residues, joined with  $\alpha$ -1, 4 linkages, but they differ from one another in the number of galacturonic acid units involved in their molecules as well as in respect of their carboxyl groups, which may either remain free or may be esterified with methyl groups. The latter two compounds (Pectins) may have a few units of such sugars as arabinose, xylose, rhamnose and galactose connected by various linkages.

Enzymes capable of degrading pectic substances are widely distributed both among the pathogenic and non-pathogenic fungi, some of which have been listed by Cochrane (1958) and Wood (1967). Different enzymes are involved in the break-down of different pectic substances, and they differ in their mode of action, the linkage which



they attack and their degradation products. Enzymatic break-down of pectic acid and pectins have been discussed below:

*Break-down of Pectic acid.* Two types of enzymatic break-down of pectic acid chains are known to date.

(1) The hydrolytic cleavage of pectic acid chains by the action of polygalacturonase (PG) enzymes are well known for many years. The polygalacturonase attacks the 1-4 glycosidic linkage and breaks the polygalacturonide chains into shorter fragments and liberates simultaneously reducing groups. Polygalacturonases have been distinguished into exo- and endo-types on the basis of their action on the pectic acid chain. The exo-polygalacturonases attack only the terminal positions of the chain and produce galacturonic acid residues, while its endo-counterpart acts at random and produces oligo-saccharides of galacturonic acid moieties.

(2) The other mechanism of enzymatic degradation of pectic acid involves transelimination and was reported by Albersheim *et al.* (1960). The enzymes catalysing such reactions were designated as transeliminases and those concerned with the degradation of pectic acid as pectic acid transeliminases (PATE). However, Bateman and Miller (1966) in their classification of pectolytic enzymes have designated them as polygalacturonate transeliminases (PGTE). These enzymes have now been found to be of wide occurrence among the fungi and their mode of action upon pectic acid chain may be illustrated as follows:

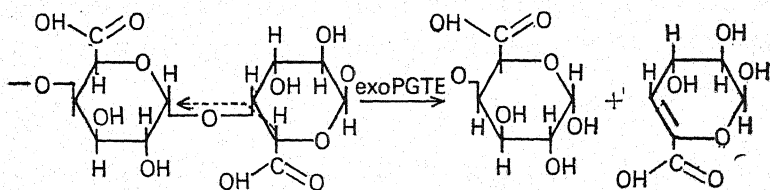


Fig. 5.4. Action of polygalacturonate transeliminase (PGTE) on pectic acid chain.

According to this scheme, the pectic acid transeliminase brings about the cleavage of the chain at 1, 4 linkage together with the transfer of the hydrogen atom from C-5 of one unit to C-1 of the adjacent unit. This results in the formation of a double bond between carbon 4 and 5 of the first unit. Transeliminases have also been further categorized into exo- and endo types (Nagel and Vaughn 1961; Bateman and Miller, 1966) on the basis of their mode of action on the pectic acid chain. The endo-PGTE causes a random cleavage of the pectic acid

chain and produces oligosaccharides with a terminal residue having a double bond between carbon 4 and 5, while the *exo*-PGTE is able to cleave off only one residue at a time, which is called 4 deoxy-L-threo-5 hexoseulosuronic acid.

**Break-down of pectins.** Enzymatic break-down of pectin compounds may take place by three different enzyme systems, *viz.* (i) Polymethylgalacturonase (PMG), (ii) pectin-methyl transeliminase (PMTE), and (iii) pectin methyl esterase (PME). PME enzyme, which is less widely distributed among the micro-organisms, attacks only the methyl ester groups of the pectin chain, and saponify them. It is unable to cause any cleavage of the pectin-chain and, therefore, the chain-length of the substrate remains unaffected. Thus, the end-products are methyl alcohol and pectinic acid chains with reduced methoxyl content. However, complete de-esterification is seldom attained, and hence conversion of pectinic acid into pectic acid is never accomplished. As illustrated below, the mode of action of PME enzyme is also hydrolytic:

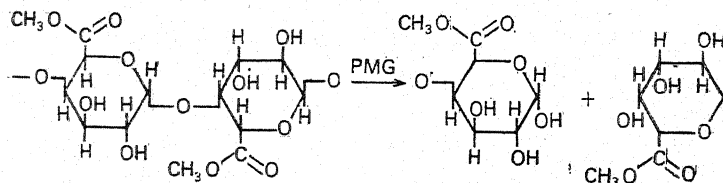


Fig. 5.4. Splitting of a polymer of methylated galacturonic acid residues by a hydrolytic enzyme (Polymethylgalacturonase).

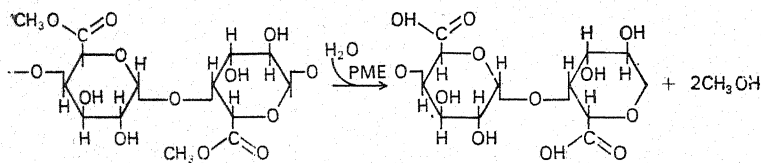


Fig. 5.5. Action of pectin methyl esterase on pectinic acid chain and removal of methyl groups.

Action of the other two types of enzymes *viz.* PMG and PMTE are similar to those of PG and PGTE respectively, which have been described earlier. Obviously the *exo*-PMG produces methylated galacturonic acid while its *endo*-counter-part liberates a series of methylated oligosaccharides of galacturonic acid. Similarly, the products

of endo-PMTE are also methylated oligosaccharides while the exo-PMTE produces a single residue at a time, viz. methyl-4-deoxy-L-threo-5-hexoseulose-uronate.

**(5) Degradation of Some Other Polysaccharides and their Derivatives**

(a) *Limit dextrin*. It may be recalled that starch-hydrolysis by  $\alpha$ -amylase remains incomplete and the resulting polysaccharide units are the dextrins. Underkofler and Roy (1951) have crystallized an enzyme from *Aspergillus oryzae*, which hydrolyzes limit dextrin and has been termed as limit dextrinase.

(b) *Inulin*. The fructosan inulin is considered to be an unbranched polymer of about 35 fructose monomers joined by  $\beta$ -2, 1 linkages. It is commonly found in plants of compositae family. For instance, tubers of dahlia and chickory are good sources of inulin. Degradation of this polysaccharide is accomplished by inulase, first discovered by Bourquelot (1893 b) from *Aspergillus niger*. Subsequent investigations (Czapek, 1922; Garren, 1938; Blank and Talley, 1941) have indicated its occurrence in many other fungi. Pigman (1943) studied some inulases produced by fungi. Although some inulase activity does occur irrespective of the carbon source employed, it has been reported by various workers (Young, 1918, Pringsheim and Kohn, 1924; Blank and Talley, 1941) that inulase activity is enhanced when inulin is employed as the carbon source. Nakatsu (1956) has suggested that the inulase may be a transfructosidase, which is also active on sucrose.

(c) *Dextran*. An inducible enzyme dextranase has been reported to be produced by a number of fungi, including *Penicillium funiculosum*, *Verticillium coccorum* and *Spicaria violacea*. (Hultin and Nordstrom, 1949; Tsuchiya *et al.* 1952). This enzyme attacks the  $\alpha$ -1, 6 glucosidic bonds of dextran and hydrolyzes it.

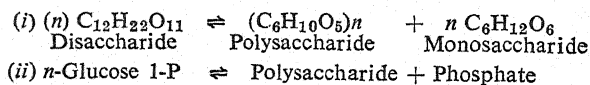
(d) *Heteropolysaccharides*. These are polysaccharides, which on hydrolysis, yield mixtures of monosaccharides and their derivatives. These include various vegetable gums, agar-agar, etc. Hydrolytic products of agar-agar are D- and L-galactose in about 9 : 1 ratio, besides sulphuric acid. Enzyme active against agar-agar viz. gelase appears to be lacking or very rare among the terrestrial fungi.

(e) *Mucopolysaccharides*. These are nitrogen containing heteropolysaccharides and are composed of N-acetyl hexosamine and hexose. In addition to these, some other mucopolysaccharides may contain hexuronic acid, sulphate or phosphate. Examples of such complex polysaccharides include chitin, hyaluronic acid, and some

unidentified cell wall constituents. Enzymes responsible for chitin degradation (chitinases) have been recorded both among the lower and higher fungi (Tracey 1955, Cantino *et al.* 1957; Reisert and Fuller, 1962; Reisert, 1972).

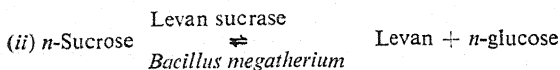
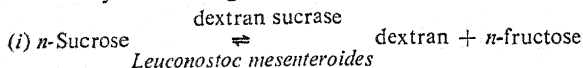
### SYNTHESIS OF POLYSACCHARIDES

A number of polysaccharides are known to be synthesized by fungi which are found either associated with the mycelium or in the culture fluid. Obviously cultures grown on carbohydrate rich media show higher activity of polysaccharide synthesis. Since Cramer's first report (1894) of a fungal polysaccharide from the spores of *Penicillium glaucum*, a number of complex carbohydrates have been reported to-date. Most of these studies have been carried out on species of *Aspergillus* and *Penicillium*, and investigations with some other fungi should help to expose the extent of their distribution among fungi in general. A list of fungal polysaccharides of known and unknown composition have been presented by Cochrane (1958), including glycogen, levan, chitin and various polyhexoses composed mostly of glucose and galactose. The process of synthesis of polysaccharides by fungi has been very little investigated, although majority of them are known to release mucilaginous substances which generally comprise polysaccharides and small amounts of proteinaceous materials, amino acids and lipids. Glucose is generally the most common constituent of polysaccharidal slime of fungi. Some other sugars like fructose, galactose, mannose, xylose, rhamnose and fucose have also been recorded. These polysaccharides appear to play a decisive role in wilt diseases of vascular plants where large polysaccharide molecules released by the pathogen in the xylem may be sufficient to cause mechanical blockage of vascular bundles. It has often been suggested that their synthesis in fungi might be following essentially the same course as in bacteria. Polysaccharide synthesis in bacteria generally takes place either through transglycosidative or phosphorylative reactions. In brief these two mechanisms may be schematized as below:



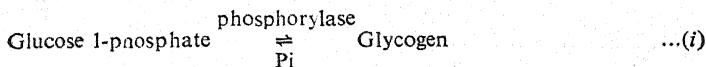
In many bacteria, however, both phosphorylases and transglycosidases are employed for the synthesis of polysaccharides. In such instances, the phosphorylase catalyzes the synthesis of a disaccharide, from which glycosyl residues are transferred by the transglycosidase

leading to the formation of polysaccharide chains. A few examples of such systems are given below:

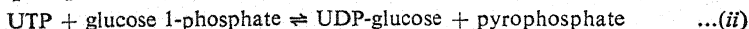


(a) *Levan*. Informations on biogenesis of this polysaccharide in fungi are rather limited. Its synthesis is known to take place in the spores of *Aspergillus sydowi*, grown on sucrose. However, levan formation does not take place, when the fungus is grown on a monosaccharide (Kopeloff *et al.* 1920) which obviously demonstrates that this also may be a transglycosidic process similar to that reported for *Bacillus megatherium*.

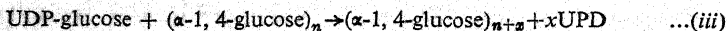
(b) *Glycogen*. Synthesis of this polysaccharide has mostly been studied with yeast glycogen. In contrast to animal system, where glycogen synthesis is well understood, the detailed mechanism of synthesis of fungal glycogen is yet to be completely elucidated. Cori and Cori (1940) demonstrated that yeast cells contained a phosphorylase enzyme which could synthesise glycogen from glucose 1-phosphate as the substrate, according to the following scheme:



However, it is now known that glucose-1-phosphate does not directly act as the substrate in this reaction. Instead, it reacts with a sugar nucleotide very similar to ATP, namely Uridine triphosphate (UTP) to form uridine diphosphate glucose (UDPG) and inorganic pyrophosphate.



Uridine diphosphate glucose (UDPG) has been detected and isolated from yeast (Leloir, 1951; Munch-Peterson *et al.* 1953). The enzyme UDP-glucose pyrophosphorylase catalyzing this reaction has also been isolated from yeast. (Algranati and Cabibe, 1960). The UDPG is referred to as "active glucose" because this glucose may under the influence of glycogen synthetase (UDP glucose-glycogen transglucosylase) may get attached to the glycogen chain by 1, 4  $\alpha$ -linkages and may elongate the chain-length of the glycogen.



The linear chain of glycogen formed in this way may grow upto 8 to 10 glucose units long. In animals and plants, as soon as the chain

attains its maximum length, a segment of three or more glucose units is transferred to a different point in the chain by the enzyme

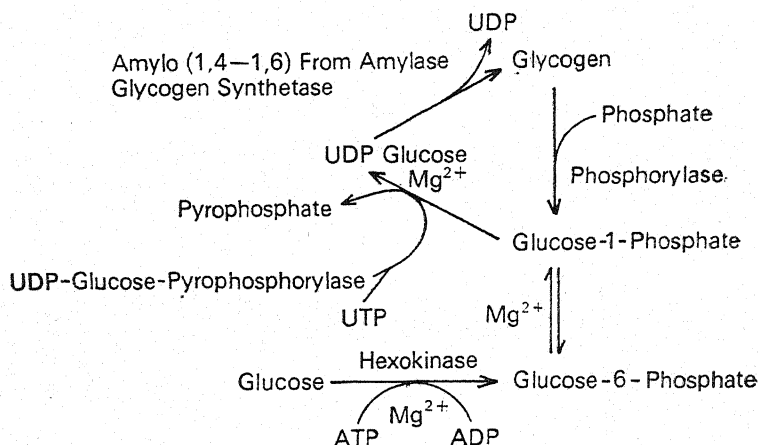


Fig. 5.6. Summary of reaction of synthesis and phosphorolysis of glycogen.

amylo (1, 4-1, 6) transglycosylase and is attached there to the 6th carbon of a sugar as a branch with  $\alpha$ -(1-6) linkage. Subsequently, both the branch as well as the open end of the chain grow in length by the addition of more UDP-glucose units by  $\alpha$ -(1-4) linkages until the chain again attains its maximum length, after which the whole sequence repeats. However, the enzyme amylo (1, 4-1, 6) transglucosylase has not yet been recorded either in yeast or filamentous fungi. Further, it may be pointed out here that the other yeast enzyme, *viz.* UDP-glucose-glycogen transglucosylase forms only  $\alpha$ -(1-4) linkages and is unable to form  $\alpha$ -(1-6) linkages. It is obvious, therefore, that this aspect of glycogen synthesis in fungi needs further attention. Moreover, in animal systems, glycogen synthesis is remarkably regulated by a specific cofactor, 3', 5' cyclic adenylic acid (cyclic Adenosine monophosphate, AMP); it may also be worthwhile to investigate the mechanism by which fungi control glycogen-synthesis.

(c) *Chitin*. Chitin is a polymer of N-acetylglucosamine and is the basic material of the cell-wall of most fungi. Evidences also suggest its possible role in the secondary wall-thickening process in fungi. However, it is interesting to note that the synthesis of this important structural component of fungal cell is yet to be fully understood. Some scattered data on the biosynthesis of chitin are available for fungi, including *Blastocladiella*, *Neurospora*, *Penicillium* and Yeast

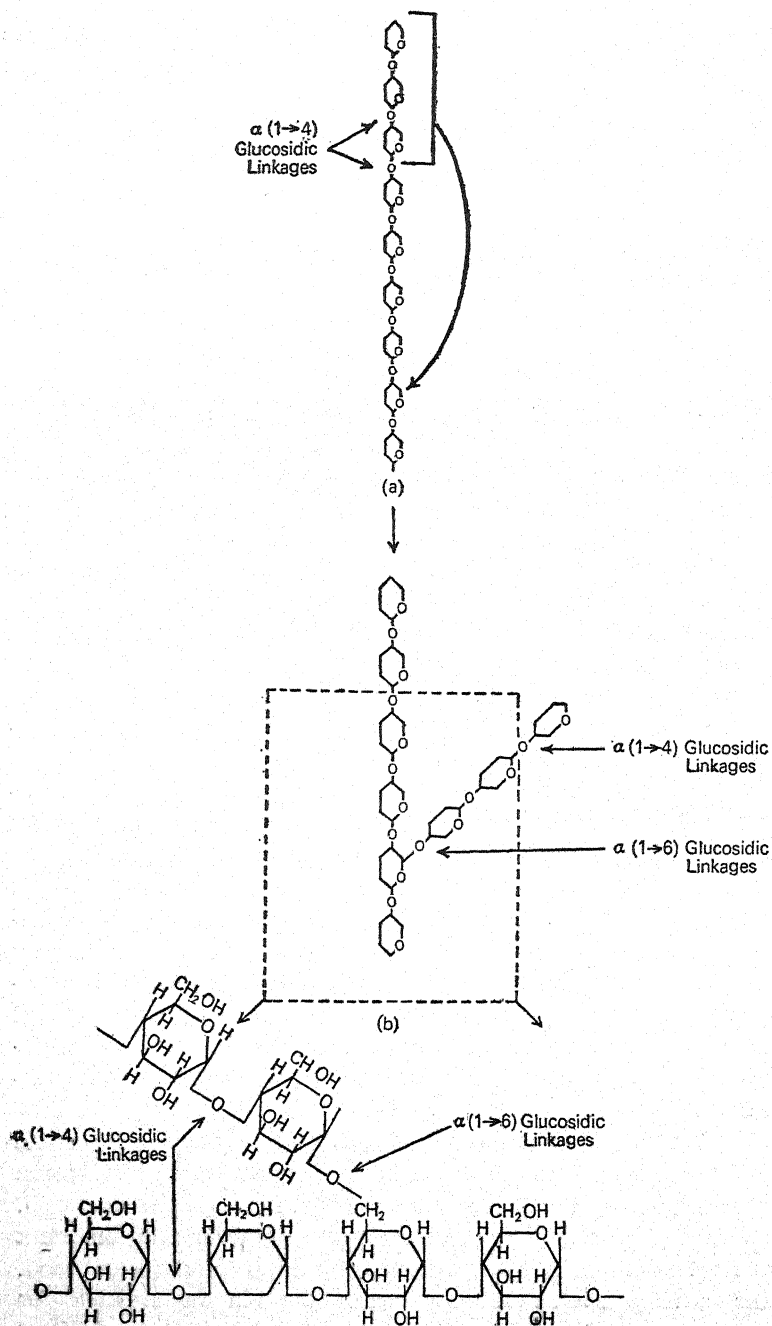
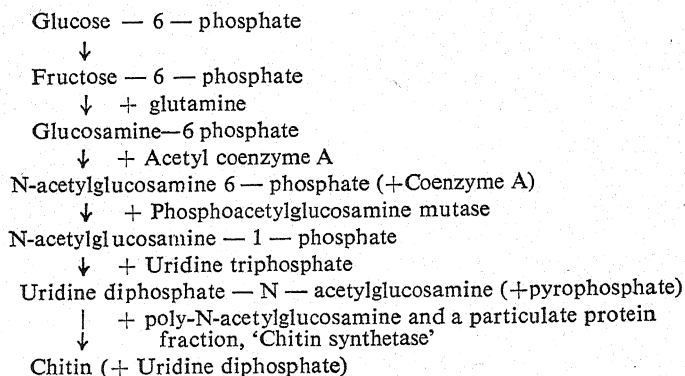


Fig. 5.7. Formation of branches during synthesis of glycogen.

(Leloir and Cardini, 1953; Blumenthal *et al.* 1955; Brown, 1955; Reissig, 1956; Davidson *et al.* 1957; Glaser and Brown, 1957 a, b; Lovett and Cantino, 1960 a). On the basis of these informations chitin-synthesis is supposed to follow a pathway, similar to the one given below:



Steps of chitin biosynthetic pathway, reconstructed on the basis of informations from different fungi; Reaction-2 in

*Neurospora*, *Penicillium* and *Blastocladiella*; 3 in

*Neurospora*, *Penicillium* and *Yesat*; 4 in

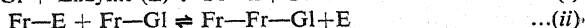
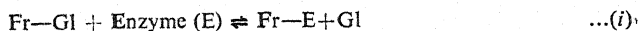
*Neurospora*, and 6 in *Neurospora*.

## OLIGOSACCHARIDES

### Degradation and synthesis of Oligosaccharides

Fungal break-down of oligosaccharides was previously visualized solely as a hydrolytic process. However, exhaustive work on carbohydrate metabolism of fungi during the last two decades (Bacon and Bell, 1953; Buston and Jabbar, 1954; Bilgrami 1956, 1963; Cochrane, 1958; Wilson and Lilly, 1958; Ghosh *et al.* 1965; Tandon, 1965; Reddy and Bilgrami, 1973) has led to the concept of transglycosidation. It is now realized that the so-called hydrolytic enzymes actually act like group-transferases and bring about transglycosidation. The concept of transglycosidation is a major break-through in the understanding of the degradation of these compounds. These group-transferases are able to transfer a sugar residue to an appropriate acceptor, which may be water or other sugars, alcohols etc. Generally, a basal carbohydrate serves as the acceptor, which results in the formation of higher oligosaccharides of varied chain length. Fischer *et al.* (1951) have presented the following scheme for the action of yeast invertase upon sucrose:





The fructose-enzyme complex shown in equation (i) is only hypothetical and is meant to explain the observed reactions. Action of transglycosidase on disaccharides typically produces new transient oligosaccharide (Equation ii).

The nature and the chain length of synthetic transient carbohydrates (oligo- and polysaccharides), appear to be dependant on the nature of the organism, concentration of the oligosaccharide in the medium, rate of its break-down and the rate of utilization of the released monosaccharides. In those cases (as in bacteria), where the synthesis of polysaccharides from oligosaccharides is quite frequent, the possibility is that the rate of break-down is rapid while the rate of consumption of the break-down sugars is possibly very slow, with the result that the transglycosidase reactions can proceed to any chain length just by linking the monosaccharide components to the original oligosaccharide in various proportions. Transglycosidase activities reported in different fungi are presented in Tables 5.2 to 5.4.

TABLE 5.2

SHOWING SOME FUNGI FORMING TRANSIENT  
OLIGOSACCHARIDES ON MALTOSE,  
CELLOBIOSE

Transglucosidase Activity	Substrate	Authors
<i>IN VITRO</i>		
		Burger and Beran (1956) Pan <i>et al.</i> (1953) Pazur and Frenzh. (1951, 52)
1. <i>Aspergillus</i> spp., <i>A. nidulans</i> group	Maltose	Mehrotra and Agnihotri (1961)
2. <i>Penicillium</i> spp., <i>P. Chrysogenum</i>	Maltose	Mehrotra and Kumar (1962) Saroja <i>et al.</i> (1955)
3. <i>Curvularia penniseti</i>	Maltose	Tandon and Chandra (1962)
4. <i>C. tuberculata</i> , <i>Drechslera austra-</i> <i>liense</i>	Maltose	Kapoor and Tandon (1970)
5. <i>Helminthosporium</i> spp.	Maltose	Reddy and Bilgrami (1973)
6. <i>Curvularia pallescens</i> , <i>Alternaria</i> <i>tenuis</i> , <i>A. citri</i>	Maltose	Hasija (1968)
7. <i>Cercosporina ricinella</i>	Maltose (High conc.)	Tandon and Chandra (1962)
8. <i>Phyllosticta carica-papayae</i> <i>P. morifolia</i> , <i>P. mortoni</i> and <i>P. sapotae</i>	Maltose	Tandon and Bilgrami (1957)

9. <i>Macrophoma allahabadensis</i>	Maltose	Kapoor and Tandon (1970)
10. <i>Botryodiplodia theobromae</i>	Maltose	Srivastava and Tandon (1969)
11. <i>Aspergillus oryzae</i>	Isomaltose	Pazur (1954)
12. <i>Colletotrichum gloeosporioides</i> , <i>C. papayae</i> , <i>Gloeosporium psidii</i> , <i>G. musarum</i>	Maltose	Ghosh <i>et al.</i> (1965)
13. <i>C. gloeosporioides</i>	Maltose	Tandon and Chandra (1962), Prasad (1965), Biharilal and Tandon (1970)
14. <i>C. capsici</i>	Maltose	Tandon and Biharilal (1968)
15. <i>Pestalotia banksiana</i> , <i>P. citri</i> and <i>P. sapotae</i>	Maltose	Tandon and Bilgrami (1957); Agarwal and Agnihotri (1970)
16. <i>Aspergillus niger</i>	Cellobiose	Barker <i>et al.</i> (1953, 55)
17. <i>Colletotrichum gloeosporioides</i> , <i>P. papayae</i> , <i>Gloeosporium psidii</i> , <i>G. musarum</i>	Cellobiose	Ghosh <i>et al.</i> (1965)
18. <i>Chaetomium globosum</i>	Cellobiose	Buston and Jabbar (1954)
19. <i>Myrothecium verrucaria</i>	Cellobiose	Kooiman <i>et al.</i> (1954)

TABLE 5.3

SHOWING FUNGI FORMING TRANSIENT OLIGOSACCHARIDES  
ON LACTOSE AND MELIBIOSE

Transgalactosidase Activity	Substrate	Authors
<i>IN VITRO</i>		
1. <i>Aspergillus oryzae</i>	Lactose	Wallenfels (1951)
2. <i>Curvularia lycopersici</i>	Lactose	Wallenfels and Bernt (1952)
3. <i>C. verruculosa</i> , <i>C. pallescens</i> , <i>C. fallax</i>	Lactose	Kakkar (1964)
4. <i>Botryodiplodia theobromae</i>	Lactose	Srivastava (1965)
5. <i>Phyllosticta carica-papayae</i> and <i>P. mortoni</i>	Lactose	Srivastava and Tandon (1969 b)
6. <i>Phyllosticta carica-papayae</i> and <i>P. mortoni</i>	Melibiose	Tandon and Bilgrami (1957)
7. <i>Botryodiplodia theobromae</i>	Melibiose	Srivastava and Tandon (1969 b)

TABLE 5.4

SHOWING SOME FUNGI FORMING TRANSIENT  
OLIGOSACCHARIDES ON SUCROSE  
AND RAFFINOSE

Transfructosidase Activity	Substrate	Authors
<i>IN VITRO</i>		
1. <i>Aspergillus</i> spp. <i>Penicillium spinulosum</i>	Sucrose	Bacon and Bell (1953); Barker <i>et al.</i> (1954), Bealing and Bacon (1953), Edelman (1954), Pan <i>et al.</i> (1953), Pazur (1952) Bealing (1953).

2. <i>Curvularia pallescens</i>	Sucrose	Hasija (1968)
3. <i>C. tuberculata</i> , <i>Drechslera australiensis</i>	Sucrose	Kapoor and Tandon (1970)
4. <i>Helminthosporium</i> spp.	Sucrose	Reddy and Bilgrami (1973)
5. <i>Macrophoma allahabadensis</i>	Sucrose	Kapoor and Tandon (1970)
6. <i>C. gloeosporioides</i>	Sucrose	Biharilal and Tandon (1970), Prasad (1965)
7. <i>C. gloeosporioides</i> , <i>C. papayae</i> , <i>Gloeosporium peridii</i> , <i>G. musarum</i>	Sucrose	Ghosh <i>et al.</i> (1965)
8. <i>Pestalotia</i> spp.	Sucrose	Tandon and Bilgrami (1958) Srivastava (1965) Bealing (1953)
9. <i>Aspergillus</i> spp. <i>Penicillium spinulosum</i>	Raffinose	
10. <i>Botryodiplodia theobromae</i>	Raffinose	Srivastava and Tandon (1969)
11. <i>Colletotrichum papayae</i> <i>C. capsici</i>	Raffinose	Ghosh <i>et al.</i> (1965) Biharilal and Tandon (1968)
12. <i>Pestalotia</i> spp.	Raffinose	Srivastava (1965)
<i>IN VIVO</i>		
13. <i>Fusarium</i> sp. (papaya fruit)	Sucrose	Ghosh <i>et al.</i> (1964)
14. <i>Macrophoma allahabadensis</i> (Guava fruit)	Sucrose	Kapoor and Tandon (1969)
15. <i>Colletotrichum gloeosporioides</i> (Mango fruit)	Sucrose	Ghosh <i>et al.</i> , (1965 b)
16. <i>Aspergillus niger</i> (Apple fruit)	Sucrose	Bisen and Agarwal (1972)

Synthesis of oligosaccharides have been reported in many fungi, some with remarkable configurations. For an instance, in galactocarolose synthesized by *Penicillium charlesii*, galactose units are probably in furanose form and are joined by 1,5 glycosidic linkages possibly in  $\beta$ -configuration. (Haworth *et al.* 1937). The general pattern of oligosaccharide synthesis is presumed to be similar to that of starch, and possibly requires appropriate sugar nucleotides and transglucosylase enzymes. Similar mechanism has indeed been elucidated during synthesis of trehalose and glycogen in fungi.

### DISACCHARIDES

Fungal degradation of some common disaccharides, like sucrose, maltose and lactose have received considerable attention. Bechamp (1858, 1864) was the first to suggest the presence of sucrose degrading enzyme in fungi which has since then received various names like sucrose, saccharase, glucosaccharase and invertase etc. The mere fact that sucrose is fairly well utilized by many fungi indicates the wide distribution of the enzyme concerned. Harter (1925) as well as Satina and Blakeslee (1928) reported the occurrence of sucrase in

some species of Mucorales, otherwise this enzyme appears to be scarce among these fungi. Subsequent studies on Mucorales (Raizada, 1957; Sarbhoy, 1965) have also furnished similar data. Similarly, Tate (1929) did not observe any sucrase activity among the five dermatophytic fungi studied by him. The common yeast *Saccharomyces cerevisiae* produces sucrase, while it is apparently absent in *Schizosaccharomyces octosporus*. The sucrase produced by yeast has been considered to be different from that produced by other fungi. The fungal sucrase was thought to be a "glucosaccharase" different from the "fructosaccharase" produced by yeast (Kuhn, 1923). Although, Neuberg and Mandl (1950) have reviewed the long-drawn controversy over the two sucraes, it has lost much of its significance after the finding that both yeast and fungus sucraes are probably transfructosidases, and there hardly remains any ground to postulate a glucosaccharase (Bealing, 1953; Edelman, 1954).

Non-hydrolytic break-down of sucrose by spores of *Myrothecium verrucaria* was suggested by Mandels (1954). Based on indirect evidences, he probably conceived of a phosphorylytic degradation of sucrose of the type found in bacteria. Although, a single kind of sucrase, viz. transfructosidase was indicated in many fungi, Bealing and Bacon (1953) postulated a possible second type in *Aspergillus oryzae*. Jermyn (1953) also concluded the presence of more than one type of sucrase in *A. oryzae* on the basis of chromatographic studies.

Since the pioneering work of Bourquelot (1883), degradation of the disaccharide maltose has been recorded in a large number of fungi. *Polyporus betulinus* (Macdonald, 1937) and some Chytrids are, however exceptions. Like sucrose, the degradation of maltose is also catalyzed by a transglycosidase enzyme in various fungi (Table 5.2). However, the occurrence of a specific hydrolase for maltose cannot be entirely ruled out because the transglycosidase activities has been observed only in a limited number of fungi. The fungal maltose differs both from its yeast counterpart (Gottschalk, 1950) as well as that from bacteria. In bacteria, the transglucosidase acting on maltose synthesizes polysaccharide. However, no such activity has yet been reported from fungi though the possibility for the same does exist.

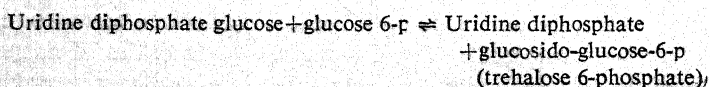
Degradation of cellobiose into its constituent glucose residues is usually attributed to enzyme cellobiase. Most of the fungi seem to possess this ability (Reese and Levinson, 1952) although much more work on this aspect is still needed. Cellobiase seems to resemble other  $\beta$ -glucosidases acting on various  $\beta$ -glucosides. Evidences for transglycosidase action of cellobiase have also been recorded from

various fungi. *Aspergillus niger* produces a cellobiase, which by transglycosidation forms new oligosaccharides. Similar transglycosidase activities have been noted in some other fungi also (Table 5.2). Cellobiose is known to be synthesized by fungi, e.g. *Ustilago zeae* synthesizes cellobiose as a component of Ustilagic acid (Lemieux *et al.* 1953).

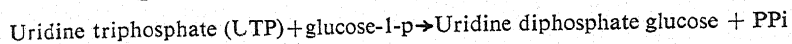
Lactose degradation by fungi is not very common. The enzyme catalyzing the hydrolysis of lactose into glucose and galactose is called lactase. It is a  $\beta$ -galactosidase and is produced by several fungal species. Wallenfels (1951) as well as Wallenfels and Bernt (1952) reported transgalactosidase activity of a lactase enzyme produced by *Aspergillus oryzae*. This enzyme could transfer the galactose residues either to alcohols, or to lactose itself forming a trisaccharide, or to water so that free galactose units were produced. However, more information is needed to make any generalisation about the transgalactosidase nature of all lactase activities.

Like lactose, melibiose is also constituted of a galactose and a glucose unit which have *alpha* glycosidic linkage while in lactose the two monosaccharides have a *beta* linkage. Very little is known about its degradation by fungi. An  $\alpha$ -galactosidase (melibiase) isolated from *Aspergillus* spp. (Thaysen and Galloway, 1930; Hofmann, 1934), and also known to occur in yeasts and higher plants brings about the cleavage of this disaccharide. Some of the records of synthesis of oligosaccharides on lactose and melibiose media are referred in Table 5.3.

The non-reducing disaccharide trehalose, commonly known as mushroom sugar, has special significance because it is synthesized by various fungi in free and appreciable quantities and it serves as a reserve carbohydrate. It is translocated and stored for future use and constitutes nearly 2 to 3 per cent of the mycelial dry matter (Reuter, 1912; Iwanoff, 1925; Takata, 1929). However, synthesis as well as break-down of this sugar is yet to be completely understood. Trehalose synthesis in yeasts is known to follow a pathway involving glucose and glucose-6-phosphate with uridine diphosphate glucose as the coenzyme. Leloir and Cabib (1953) have shown the presence of an enzyme in yeast, which can transfer glucose from UDP-glucose to glucose-6-phosphate to form trehalose-6-phosphate. The pathway may be schematized as follows:



Uridine diphosphate glucose is regenerated as follows:



Thus, uridine diphosphate glucose serves here as a monosaccharide unit donor. However, whether the same scheme of trehalose synthesis is operative in filamentous fungi also, is not yet definite.

Similarly, the break-down mechanism of this compound is also little understood. Although it is an  $\alpha$ -glucosido-glucose, yet trehalose is not attacked by the usual  $\alpha$ -glucosidase enzymes. Instead, a specific enzyme designated as trehalase is able to affect its degradation, and is commonly found in animals, plants and fungi (Gottschalk, 1950). Work on fungal trehalase (Czapek, 1922; Satina and Blakeslee, 1928; Willstaedt and Borggard, 1946; Mager, 1947) has amply indicated its common occurrence among fungi. Recently, Williams and Niederpruem (1968) have isolated trehalase enzyme from the mycelial extract as well as culture filtrate of *Schizophyllum commune*, growing on trehalose as well as glucose containing media.

**Higher oligosaccharides.** The trisaccharide raffinose is a by-product of beet sugar manufacture and on complete hydrolysis produces equivalent amounts of glucose, fructose and galactose. Most of the fungi studied so far have been found to cause an enzymatic break-down of this sugar. Mostly, such a break-down is attributed to a sucrase, which splits this trisaccharide into melibiose and fructose, but for its complete break-down both sucrase and melibiase are essential. Action of melibiase alone, produces sucrose and galactose. Synthesis of transient oligosaccharide due to transglycosidase activity has been recorded in several fungi (Table 5.4).

Two other trisaccharides have been assayed as fungal substrates, viz. melezitose and gentianose. Bourquelot (1898) as well as Bourquelot and Herissey, (1901) reported that an enzymatic preparation from *Aspergillus niger* completely hydrolyzed gentianose, and was later found to contain a  $\beta$ -glucosidase enzyme, which attacked the gentiobiose unit of the trisaccharide (Bourquelot and Herissey, 1902). Similarly, complete hydrolysis of melezitose was achieved by an enzyme acting upon its disaccharide moiety turanose. Bealing (1953) as well as Kuhn and Grundherr (1925) have reported that several fungi are capable of hydrolyzing (at least partially) melezitose.

A single tetrasaccharide viz. stachyose has found limited applicability for enzymatic studies on fungi. Composed of galactose, glucose and fructose (2 : 1 : 1), stachyose is probably degraded by the action of a sucrase and an  $\alpha$ -galactosidase. Bealing (1953) and Tanret (1903)

has reported the enzymatic splitting of this oligosaccharide by preparations from *Penicillium spinulosum* and some *Aspergilli*.

## SOME OTHER GLYCOSIDES

### Other Glycosides

Glycosidic linkage may be formed either between two sugar units only or between a sugar and a non-sugar moieties. The non-sugar moiety is called aglycon. Glycosides with an aglycon component have been little investigated pertaining to their metabolism by fungi, and those investigated are either *alpha* or *beta* isomers of glucosides or mannosides.

The enzymes catalyzing the degradation of glucosides are designated as glucosidases and according to the *alpha* and *beta* isomers of the substrate, the specific enzymes are referred as  $\alpha$  and  $\beta$ -glucosidases. The  $\beta$ -glucosidase activity has been found to occur in almost all the fungi and actinomycetes investigated so far (Tate, 1929; Bose, 1939; Saksena and Bose, 1944; Mehrotra, 1949; Hofmann and Latzko, 1950). However, certain yeasts (other than the lactose fermenting) are known to lack the enzyme  $\beta$ -glucosidase and, therefore, are unable to ferment  $\beta$ -methyl glucoside. Davis *et al.* (1953) found that *Fusarium oxysporum* var. *lycopersici* secreted this enzyme in its host in detectable quantity. However, in culture media this may not be always detected (Reese and Levinson, 1952).

$\beta$ -glucosidase from various sources exhibit different behaviour, and it is now considered more likely that  $\beta$ -glucosidase either comprises a group of enzymes (Viebel, 1950) or it consists of different components (Jermyn, 1952, 53). Earlier Miwa *et al.* (1937) had shown that the  $\beta$ -glucosidase obtained from *Aspergillus niger* and *A. oryzae* caused the degradation of some synthetic  $\beta$ -glucosides at different rates. Similar variations in the behaviour of  $\alpha$ -glucosidases obtained from various sources, have also been observed. However, information on the activities of the  $\alpha$ -glucosidases from different sources are too fragmentary to draw any definite conclusion. An  $\alpha$ -glucosidase, maltase (of fungi) exhibited very little activity on methyl- $\alpha$ -glucoside. This substrate was slowly attacked by an enzymatic preparation from *Aspergillus niger* also, although methyl- $\beta$ -glucoside was very rapidly degraded by this preparation (Dox and Neidig, 1912). This fungus was induced to produce an  $\alpha$ -galactosidase when grown on methyl- $\alpha$ -galactoside. (Pottevin, 1903).

*A. niger* has also been reported by Herissey (1921) to form an  $\alpha$ -mannosidase when grown on methyl- $\alpha$ -mannoside. Another report

on mannoside degradation has come from Hockenhull *et al.* (1954). They isolated an enzyme from *Streptomyces griseus*, which hydrolysed the synthetic methyl- $\alpha$ -mannoside. The same enzyme transforms mannosidostreptomycin into streptomycin (Perlman and Langlykke, 1948).

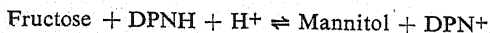
### Sugar Derivatives

Fungal metabolism of only a few carbohydrate derivatives, including some sugar-alcohols, sugar acids, amino-sugars etc. has been studied so far.

(a) *Sugar alcohols*. Simple sugars are converted into their corresponding alcohols by reduction of their aldehyde or Ketone group. These are widely distributed in nature and are utilized by most of the fungi, although they appear to be inferior source than their corresponding sugars. Some of these alcohols are known to be synthesized by various fungi.

Mannitol, a derivative of the sugar mannose, is known as a fungal metabolite since the beginning of this century. Subsequent investigations with fungi of diverse groups have also indicated its wide occurrence among fungi. Mannitol has been detected in the sporophores of Basidiomycetes and Ascomycetes (Bourquelot, 1893 a; Zellner, 1907; Czapek, 1922); mycelium of *Aspergillus elegans* (Gregoire, 1949) and *Rhizopus japonicus* (Lim, 1935) as well as in sclerotia (Ergle, 1948) and spores of some fungi. (Zellner, 1910, Sumi, 1928; Wain and Wilkinson, 1946).

Mannitol may be formed in the culture medium in appreciable quantities from pentoses, hexoses and glycerol (Coyne and Raistrick, 1931; Yamasaki and Simomura 1937). Conversion of glucose to mannitol was quite rapid (from 20 to 50% of the glucose consumed) in case of *Aspergillus* sp. (Birkinshaw *et al.* 1931) and *Byssoschlamys fulva* (Raistrick and Smith, 1933). Mannitol is, therefore, considered to serve as the reserve food material of fungi, although its biosynthesis has not been studied so far. However its formation by the reduction of fructose or a fructose-phosphate appears to be quite reasonable. Cochrane and Albrecht (cited by Cochrane, 1958) found that fructose may be reduced to mannitol under the influence of *Stachybotrys atra* extract. This reversible reaction may be schematized as follows:



It is obvious that a hydrogen donor is essential for the reaction to proceed the synthetic way. Enhanced synthesis of mannitol under



Limited supply of oxygen (Birkinshaw *et al.* 1931) also suggests that mannitol synthesis involves some reducing reactions. However, more investigations are essential for elucidation of the complete process.

Fungi are also known to synthesize some other sugar alcohols, including D-sorbitol, D-volemitol, D-arabitol, L-erythritol, etc. and accumulate them. Sometimes, quite an appreciable amount of the metabolized carbon is transformed into sugar alcohols. For instance, Birkinshaw *et al.* (1948) recorded that L-erythritol constituted nearly 13 per cent of the mycelial dry-weight of *Armillaria mellea*. *Boletus bovinus* synthesizes D-sorbitol (Lippmann, 1912). Reports of dehydrogenation of some sugar alcohols into their corresponding sugars are also available. Several Basidiomycetes transform sorbitol to L-sorbose (Vitucci *et al.* 1946). Similarly, Goepfert and Nord (1942) found that intact cells of *Fusarium lini* oxidize erythritol to erythrulose.

(b) *Amino sugars*. Amino sugars owe their significance in fungal metabolism chiefly on two accounts. Primarily, because N-acetyl derivative of an amino sugar, 2-amino-D-glucose (glucosamine) is a basic constituent of chitin, the cell-wall material of most of the fungi, and secondly due to the frequent presence of some amino-sugars as a component of antibiotics *e.g.* cordycepin from *Cordyceps militaris* (Bentley *et al.* 1951).

Although, only limited information is available on the synthesis of glucosamine, yet it seems quite reasonable to suggest that this takes place through the conversion of glucose to glucosone. In fact, synthesis of glucosone is known to occur in *Aspergillus parasiticus*, under certain circumstances (Bond *et al.* 1937). However, Leloir and Cardini (1953) have reported that in *Neurospora crassa* synthesis of glucosamine takes place through a transamidation process, which may be schematized as below.

Hexose 6-phosphate + glutamine  $\rightarrow$  glucosamine-6-phosphate + glutamic acid.

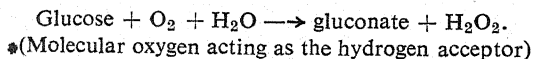
Blumenthal *et al.* (1955) have indicated that the hexose phosphate participating in the formation of glucosamine phosphate through transamidation reaction, may be a fructose-6-phosphate.

(c) *Sugar acids*. These are derivatives of simple sugars as indicated in Chapter IV. They may be of three different kinds, *viz.* uronic acids, glyconic acids and glycaric acids. Although several sugar acids are now known to be formed by fungi yet they have been little investigated with regard to their synthesis. The only exception is gluconic acid, which has been investigated intensively due to its industrial importance, as well as being the only sugar acid synthesized by fungi in appreciable quantity. Gluconic acid synthesis has

indeed been reported from a variety of fungi, since it was first discovered by Molliard (1922) in *Aspergillus niger*. Cochrane (1958) has enlisted several fungi which produce different sugar acids.

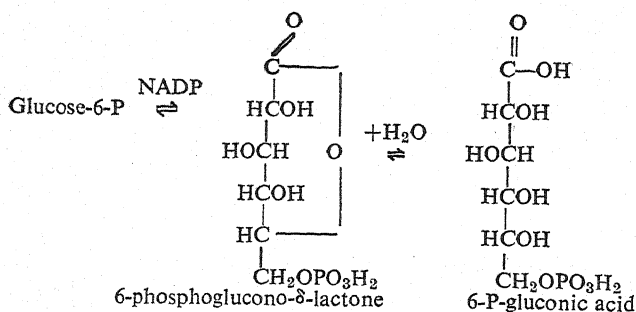
Conversion of glucose to gluconic acid is known to take place through phosphorylative as well as non-phosphorylative pathways of respiration.

The chief characteristic of the non-phosphorylative pathway is the direct oxidation of glucose, to gluconic acid, without its prior phosphorylation. Such an oxidation of free glucose is known to occur under the influence of an enzyme, first discovered by Muller (1926, 28) in *Aspergillus niger* and referred to as notatin or glucose oxidase. Subsequent investigations by Bentley and Neuberger (1949), with the help of isotopic oxygen has revealed that this enzyme is actually a dehydrogenase and, therefore, may well be referred as glucose aerodehydrogenase. This enzyme differs from its bacterial counterpart found in *Pseudomonas fluorescens* (Wood and Schwerdt, 1953) as well as from the glucose dehydrogenase of liver (Harrison, 1931) in being a flavoprotein. *Penicillium chrysogenum* is known to form flavoprotein enzyme with high specific activity towards  $\beta$ -D-glucose. The overall process of non-phosphorylative pathway of glucose oxidation may be schematised as below:

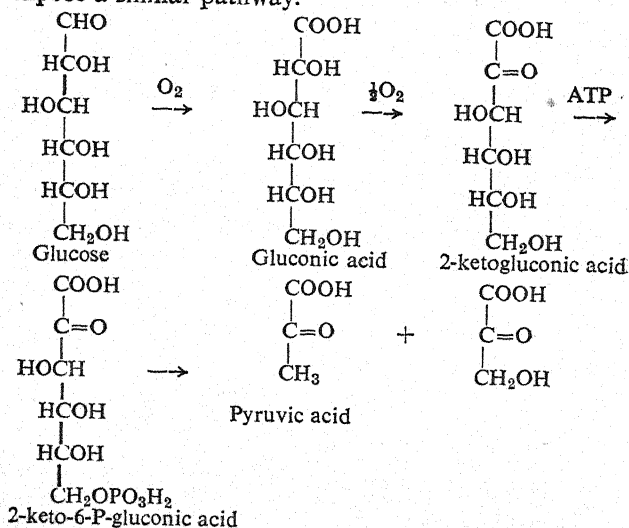


Possibilities of existence of other similar aerodehydrogenases have also been indicated (Knöblock and Mayer, 1941), which might be controlling the synthesis of other hexonic acids, like mannonic acid and galactonic acid by *Aspergillus niger*, (Knöblock and Mayer, 1941) and lactobionic acid by *Penicillium chrysogenum* (Cort *et al.* 1956). However, synthesis of gluconic acid without the occurrence of a glucose aerodehydrogenase enzyme has also been reported in *Mucor racemosus* and *Dematium pullulans* (Franke and Deffner, 1939), and hence no generalisation regarding participation of such enzymes may safely be made.

Synthesis of gluconic acid through a phosphorylative pathway is known to occur as a preliminary step of Entner-Doudoroff (ED) as well as pentose phosphate pathways of respiration. In this step, glucose-6-phosphate is oxidized to 6-phosphogluconic acid under the influence of dehydrogenases as well as the coenzyme NADP. The reactions may be shown as under:



Further metabolism of gluconic acid in fungi, is yet to be understood. Two possibilities have been indicated in this regard, one involving phosphorylation of the gluconic acid and the other a continuation of the non-phosphorylative pathway, similar to that traced for *Pseudomonas* and *Acetobacter* (Thimann, 1955; Wood, 1955; Hollman and Touster, 1964). The second probability has aroused much interest due to the detection of 2-keto-gluconic acid in the culture fluid of *Penicillium brevi-compactum*. As is shown below 2-keto-gluconic acid is a product of non-phosphorylative metabolism of gluconic acid in bacteria and, therefore, further work in fungi may also expose a similar pathway:



Formation of some other sugar acids, like glucuronic acid, saccharic acids etc. have been reported from *Aspergillus niger* and that of pentonic acids from *Fusarium lini* but the reports are of very preliminary type, and further work is essential for the understanding of their metabolism in fungi.

## CARBON METABOLISM—II

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That fungi are capable to utilize a variety of carbon compounds other than carbohydrates, was referred in Chapter IV. The non-carbohydrate compounds metabolized by fungi include both aliphatic and aromatic compounds. However, lipids and organic acids are obviously the principal non-carbohydrate substrates metabolized by fungi and they have been given their due treatment. Metabolism of steroids, terpenoids and aromatic compounds have also been discussed in brief. Several other carbon containing metabolites of fungi, as well as their biosynthesis have been included in Chapter XVI and therefore they have been skipped over here.

### 576-68 ORGANIC ACID METABOLISM

Chemistry as well as nutritional value of some organic acids as carbon sources for fungi were discussed in chapter IV. Synthesis of certain TCA cycle acids and amino acids have been described in succeeding chapters. Here we confine only to aliphatic, non-fatty organic acids and their metabolism by fungi. Fatty acid metabolism has been taken up later in this chapter.

Synthesis of organic acids by fungi was first confirmed by the classical studies of Wehmer during 1896-97. Since then, a large number of reports have appeared, and acids belonging to different groups have been recorded as metabolic product of fungi. Some of the important contributors in this field have been Bernhauer, Birkinshaw, Butkewitsch, Chrzaszoz, Cohen, Neuberg, Raistrick and their co-workers. However, much remains to be understood regarding the biosynthetic processes leading to the formation of these acid in a fungal cell. Most of the modern concepts regarding the mechanism of their synthesis presuppose the glycolytic conversion of the carbohydrate substrate into pyruvic acid from which only these acids are believed to be produced by varied transformations. Whether such

indirect pathways operative in a fungal cell are very much responsible for production of organic acids in good amount is yet to be ascertained.

### Oxalic Acids

Crystals of oxalic acid were first observed by Wehmer (1891) in the mycelia as well as the culture medium of *Aspergillus niger* and he considered this acid as the product of metabolism of fungi. Currie and Thom (1915) named a species of *Penicillium* as *P. oxalicum* because that fungus was able to produce oxalic acid. Now many fungi are known to synthesize this acid. Agaricales very commonly accumulate oxalic acid, while members of Aspergilli and Penicillia produce this acid in largest quantities. Oxalic acid production is also widespread in the basidiocarps of Polyporales, in *Sclerotium delphinii*, etc. Synthesis of oxalic acid is favoured by high glucose concentration, adequate aeration and high initial pH.

Butkewitsch and Fedoroff (1930) suggested two possible sequence of reactions for conversion of acetate to oxalate by *Mucor stolonifer*:

- (a) Acetic acid  $\rightarrow$  Glycolic acid  $\rightarrow$  Glyoxalic acid  $\rightarrow$  Oxalic acid
- (b) Acetic acid  $\rightarrow$  Succinic acid  $\rightarrow$  Fumaric acid  $\rightarrow$  Malic acid  $\rightarrow$  Ketosuccinic acid  $\rightarrow$  Oxalacetic acid.

Investigation pertaining to biosynthesis of oxalic acid has since been carried further but none of the pathways proposed has been fully established (Allsopp, 1937, 1950; Nord and Weiss, 1951). Bomstein and Johnson (1952) concluded from his studies with  $^{14}\text{C}$ -labelled compounds that in *Aspergillus niger* oxalate is possibly produced from oxaloacetate. The enzyme oxaloacetate hydrolase catalyzes this conversion and requires  $\text{Mn}^{2+}$  (Hayaishai *et al.*, 1956). Some other reports also suggest conversion of oxaloacetate into oxalic acid (Foster, 1951; Lewis and Weinhouse, 1951 b; Cleland and Johnson, 1956) but further investigation is necessary to confirm these findings.

### Citric Acid

In the year 1893, Wehmer observed the production of citric acid by a fungus which he named as *Citromyces*. Later this physiological characteristic was observed in many Aspergilli and Penicillia and, therefore *Citromyces* with about 20 species was merged with *Penicillium*. It is now realised that citric acid is produced by all the aerobic fungi as a TCA cycle product, but in appreciable quantities the acid is accumulated only by a few fungal species or strains, under appro-

priate conditions, like high (15-20%) glucose concentration, and relatively low pH (Ca.2.0). Maximum accumulation of the acid occurs when the primary growth processes are retarded. It has also been reported that high-yielding strains are slow-growing, while low-yielding strains are fast-growing (Gardner *et al.* 1956; Erkama *et al.* 1949). Such strainal variations suggest that the process involves some genetic mechanism, although the concerned locus has not been located. The slow growth of the high yielding strain has been suggested to be on account of low or no activity of the enzyme isocitrate dehydrogenase (Ramakrishnan *et al.* 1955).

Mechanism of biosynthesis of citric acid by fungi has received considerable attention, and a number of possible pathways have been suggested. However, none of the schemes proposed has been fully accepted. Citric acid is known to be synthesized from 2 to 12-carbon compounds, and any acceptable biosynthetic pathway must account for all these. There are some other observations also, like high yields (upto 100%) of citric acid from sugars, which are hard to explain by any theory proposed so far. Different pathways for citric acid synthesis visualize its formation from glucose, Raistrick and Clark, 1919 (Butkewitsch 1924), acetic acid, (Chrzaszcz and Tiukow, 1930), malic acid (Ciusa and Brull, 1939) and various other compounds. All these pathways have been discussed by Walker (1949) as well as Foster (1949) and need not be detailed here.

It is now realised that citric acid synthesis by fungi is somehow related to the TCA cycle. Cochrane (1958) has regarded accumulation of citric acid as an unusual modification of the TCA cycle, under genetic and environmental control. Lewis and Weinhouse (1951) working with labelled acetate concluded that citric acid accumulated due to faulty operation of the Krebs cycle. Ramakrishnan *et al.* (1955) concluded that interruption of the Krebs cycle at isocitric dehydrogenase and aconitase enzyme levels, with a simultaneous increase in the activity of citric acid condensing enzyme resulted in the accumulation of citric acid. They also observed that during early incubation period when citric acid was not accumulating, all the enzymes of TCA cycle, including isocitric dehydrogenase and aconitase were active.

Another postulate, which has a wide support and is based on data obtained from isotopic studies considers that citric acid is formed as a result of condensation of 2-carbon (may be acetate) and 4-carbon (a dicarboxylic acid) compounds. The dicarboxylic acid may in turn be formed by condensation of two 2-carbon compounds or one

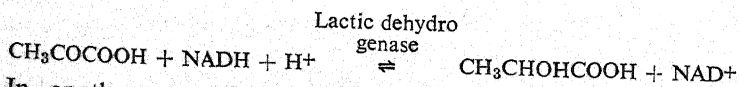
1-carbon compound and a 3-carbon compound (Foster and Carson, 1950).

Shu, Funk and Neish (1954), have obtained data from isotopic studies to suggest that in *Aspergillus niger*, 37 to 40 per cent citric acid is formed from recycled C-4 dicarboxylic acid, 40% of which in turn is produced by a  $C_2 + C_2$  condensation and the rest 60% by  $C_1 + C_3$  condensation.

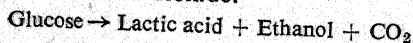
### Lactic Acid

Lactic acid biosynthesis by *Mucor rouxii* was suggested as early as in 1894 by Eijkmann which was later confirmed by Chrzaszcz in 1901. Commercial production of lactic acid by a fungus, then designated as "*Lactomyces*" (now believed to be a *Rhizopus* sp.) was undertaken by Boullanger in 1899. Since then a number of fungi belonging almost exclusively to Phycomycetes, have been found to synthesize this acid. Only a few outside this class have been reported to form lactic acid including *Botrytis cinerea* (Gentile, 1954) *Monilia* spp. (Miyaji, 1930; Sakaguchi and Inoue, 1940). Cochrane (1958) has given a list of fungi producing lactic acid, while earlier literature on this aspect have been reviewed by Foster (1949), Lockwood *et al.* (1936) and Ward *et al.* (1936, 1938).

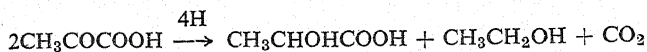
As indicated in Chapter VII both homo and heterolactic fermentation have been observed in fungi. Lower Phycomycetes like *Allomyces arbuscula*, *Blastocladia pringsheimii* and various Leptomitales, anaerobically convert 85% to 100% of glucose consumed to lactic acid. During this process no ethyl alcohol is produced, and aerobic conditions have no stimulating effects on lactic acid accumulation. With the recent detection of a NAD-linked, D-lactic dehydrogenase enzyme in *Blastocladia* (Cantino and Lovett, 1960; Gleason *et al.* 1966) as well as in several Leptomitales, it seems now fairly definite that these fungi synthesize lactic acid in the same manner as it takes place in animal muscle. Glucose is converted to 2 molecules of pyruvic acid through Embden-Meyerh of pathway which is anaerobically transformed into lactic acid:



In another group of fungi, mainly represented by species of *Rhizopus* lactic acid synthesis is associated with an equimolar yield of ethyl alcohol and carbon-dioxide.



Data obtained from isotopic studies on  $^{14}\text{C}$ -1 or  $^{14}\text{C}$ -3, 4 glucose catabolism by *R. oryzae* (Gibbs and Gastel 1953) suggest that all the three products arise from a common pool of triose phosphate. The  $^{14}\text{C}$ -data obtained by Carson *et al.* (1951) had earlier indicated that pyruvic acid is directly reduced to lactic acid in the following manner.



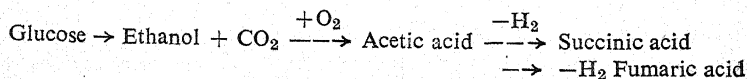
However, it is not yet clear how this step is brought about, nor the manner in which the 1 : 1 ratio of lactic acid and ethanol is maintained. Further, the enhanced lactate synthesis under aerobic conditions by these fungi is not understood and whether pyruvic acid or ethanol is the source of increased quantity of lactic acid is also not clear. Waksman and Foster (1939) found that under aerobic conditions, lactic acid synthesis increased with a simultaneous decrease in the quantity of ethanol. Irrespective of the source of the enhanced lactic acid, it seems that some 4-carbon acid serves as an intermediate (Carson *et al.* 1951).

### Fumaric Acid

Ehrlich (1911) demonstrated the synthesis of this unsaturated four-carbon dicarboxylic acid by *Rhizopus nigricans*. Later Wehmer (1918) reported high yields (60-70%) of fumaric acid from an *Aspergillus* species which he designated as *A. fumaricus*. Interestingly, this species gradually lost its capacity to synthesize fumarate. Capacity to synthesize fumaric acid is now known to belong to a number of phycomycetous genera listed by Prescott and Dunn (1959). Besides the Phycomycetes, this ability has been recorded in *Penicillium griseo fulvum* and *Aspergillus fumaricus* only. Birkinshaw *et al.* (1942) reported that *Penicillium resticulosum* forms fumaryl-DL-alanine in a glucose rich medium.

Biosynthesis of fumaric acid is favoured by a high glucose concentration and an appreciable amount of zinc. Besides its oxidative formation in the TCA cycle, three possible routes of formation of this acid in bulk have been suggested:

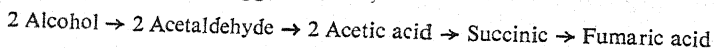
(1) Gottschalk (1926) as well as Butkewitsch and Fedoroff (1929) suggested that fumaric acid may arise in an alcoholic fermentation as follows:



Foster (1949) has drawn support for this sequence from the common

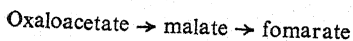


observation that 2-carbon compounds remain associated with fumarate. The sequence he suggested was,



which involved the condensation of two 2-carbon fragments by the Thunberg-Wieland reaction. Disappearance of C-2 compounds from culture solution and concomitant formation of C-4 acids has also been taken as evidence in support of this sequence. Further, a number of workers have advanced both chemical and isotopic data (Foster and Carson, 1950; Barron and Ghiretti, 1953) to provide support for this pathway in fungi. However, the distribution of isotopic carbon somewhat complicates the interpretation of the data, and the applicability of the Thunberg reaction to this pathway has been questioned.

(2) Foster and Davis (1948) reported that in *Rhizopus nigricans* fumaric acid is produced by an anaerobic sequence. This sequence is thought to involve reductive carboxylation of pyruvic acid. The incorporation of carbon dioxide into the pyruvic acid molecule may result in the formation of oxaloacetic acid or under the influence of the malic enzyme it may lead directly to the formation of malic acid. Further steps leading to the synthesis of fumaric acid are as follows:



Incorporation of carbon dioxide into the fumarate molecule via this sequence has been demonstrated with labelled  $^{14}\text{CO}_2$ , and it seems that this pathway plays a major role in fumarate synthesis.

(3) The third alternative pathway of fumarate accumulation involves the cleavage of the isocitric acid into succinic and glyoxylic acids (Olson, 1954), and transformation of succinic acid into fumaric acid, obviously under the influence of succinic dehydrogenase.

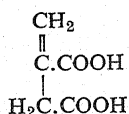
This sequence involves a part of the glyoxylate shunt, which is now known to supplement the TCA cycle in fungi and to provide a mechanism for the synthesis of C<sub>4</sub>-dicarboxylic acids. Its role in fumarate accumulation has been supported by Foster *et al.* (1949).

From the above discussion, it is apparent that three other 4 carbon dicarboxylic acids, viz. Oxaloacetic, malic and succinic acids, are closely related to fumaric acid and the four are easily interconvertible. Oxaloacetic acid is rather unstable and seldom accumulates. Similarly succinic and malic acids have also been scarcely reported to be synthesized in any large amount. *Blastocladia pringsheimii* synthesizes succinic acid in amount approximately 10% of glucose consumed. A number of other fungi produce this acid during oxida-

tive break-down of sugars. Malic acid which is now known to be produced by a number of saprophytic fungi, was first recorded in traces from sucrose fermentation by *Aspergillus fumaricus* (Wehmer, 1928). Later Raistrick *et al.* (1931) recorded malic acid synthesis during glucose utilization by several fungi.

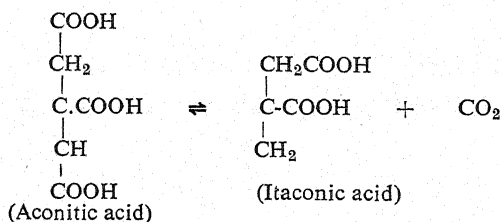
### Aconitic, Itaconic and Itatartaric Acids

Kinoshita (1931 a,b) for the first time isolated itaconic or methylene succinic acid from an *Aspergillus* sp., which he named as *A. itaconicus*. This unsaturated dicarboxylic acid has the formula,



and due to the presence of a double bond, two carboxyl groups and the allylic methylene group, this acid is quite reactive. A few strains of *Aspergillus terreus* have been reported to form itaconic acid in commercially significant amount (Calam *et al.* 1939; Moyer and Coghill, 1945; Lockwood and Reeves, 1945).

Aconitic acid, which is an intermediate of TCA cycle gives rise to itaconic acid under the catalytic influence of cis-aconitic decarboxylase (Bentley and Thiessen, 1957);



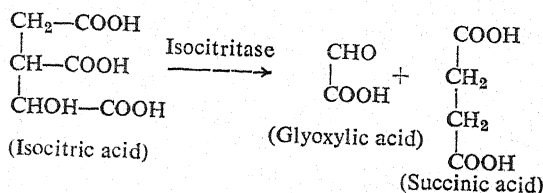
This step has been regarded as a diversion in the normal catabolism of glucose (Cochrane, 1958). The optimum conditions for the synthesis of itaconic acid include low pH range (1.9 to 2.3) and high concentration of magnesium sulphate (Lockwood and Reeves, 1945). Recently Mehrotra and Tandon (1970) have confirmed that magnesium sulphate supports itaconic acid production by *A. terreus*. None of the three other salts of magnesium, they used, supported itaconic acid synthesis. Further, they observed that sulphate ions were more effective than the magnesium ions, the latter probably helps in antagonising the toxicity of aluminium ions only.

Attempts to obtain mutants of *Aspergillus terreus* for higher production of itaconic acid have been made by ultraviolet irradiation of

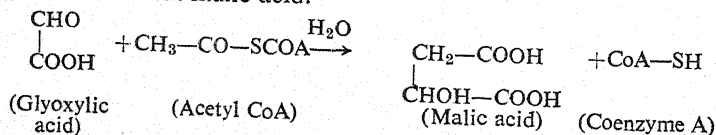
conidia (Raper *et al.* 1945). During a similar investigation, Stodola *et al.* (1945) obtained a mutant strain NRRL 265.S14, of *A. terreus* which produced besides itaconic acid ( $C_5H_6O_4$ ), a hydroxyl acid, *viz.* itatartaric acid ( $C_5H_8O_6$ ) and its lactone. From its chemical formula, it is obvious that it is related to itaconic acid and may be its derivative.

### Glyoxylic Acid

In a number of fungi and bacteria, glyoxylic acid synthesis is now known to take place during the glyoxylate shunt, which provides for a second point of entry of acetate into the TCA cycle. The enzymes isocitritase (Olson, 1959) and malate synthetase (Dixon *et al.* 1960), which are concerned in glyoxylic acid metabolism, have been purified from yeast and evidences for their presence have been recorded in a number of fungi (Kornberg and Collins, 1958; Collins and Kornberg, 1960; Gottlieb and Ramachandran, 1960; McCurdy and Cantino, 1960; Frear and Johnson, 1961; Turian 1961). The isocitritase brings about a cleavage of the isocitric acid molecule to give rise to glyoxylic acid and succinic acid:



The glyoxylic acid then acts as a carrier of two carbon acetate fragments and under the influence of malate synthetase combines with acetyl CoA to form malic acid:



The resulting malate as well as the succinate, (produced in the previous step) enter the TCA cycle, and provide a second source for the synthesis of oxaloacetic acid. However, the isocitritase is an adaptive enzyme and its formation takes place only when there is a shortage of glucose or succinate (Krebs and Lowenstein 1960). Probably on this account only glyoxylic acid synthesis has been reported more frequently during metabolism of non-glucose substrates, like acetate (Bernhauer and Scheuer, 1932; Nord and Weiss, 1951), ethanol

(Chrzascz *et al.*, 1932) and citrate (Challenger *et al.* 1927 a).

### Other Acids

Some other acids have been detected in fungi, but less frequently and are obviously produced in traces by fungi. These include acetic, formic, tartaric, glutaric, glutaconic, dimethyl pyruvic, and ethylene oxide  $\alpha$ ,  $\beta$ -dicarboxylic acids. Acetic and formic acids are volatile acids and are known more as bacterial products than as fungal metabolites. Of the two, acetic acid is more relevant to fungal metabolism, in view of its significant role as acetate and acetyl-coenzyme A and, therefore, its omnipresence in fungal mycelium may be safely presumed even though in several cases, it may be difficult to demonstrate the same. However, many fungi have been recorded to produce acetic acid in traces (Cochrane, 1958) or in larger amounts (Lockwood *et al.* 1938). In others, its presence has been indicated by isotope dilution procedure (Martin *et al.* 1953). Some of the fungi have been shown to produce acetate during metabolism of pentose (Gibbs *et al.* 1954), ethanol (Nord, 1940; Perlman, 1949) and glycine (Woodruff and Foster, 1943) while others like *Merulius niveus* (Barron and Ghiretti, 1953), *Penicillium chrysogenum* (Hockenhull *et al.* 1954) and *Zygorhynchus moelleri* (Mortimer and Johnson, 1952) are known to utilize acetate supplied from outside. *Endoconidiophora moniliformes* (Gordon, 1950) and *Penicillium digitatum* (Birkinshaw *et al.* 1931) have been recorded to form ethyl acetate.

That formic acid participates in the metabolism of fungi was indicated by data obtained by Jefferson and Foster (1953) from their isotopic studies, as well as from some nutritional studies (Chrzascz and Zakomorny, 1935; Harrold and Fling, 1952). Otherwise, accumulation of this acid during carbohydrate utilization has not been observed. Its formation has of course been reported in several fungi during metabolism of pyruvic and lactic acids (Chrzascz and Schillak, 1936; Sakaguchi *et al.* 1942). Formic acid synthesis and utilization is presumed to take place according to the following sequence, although further careful studies are needed to substantiate them in fungi.

(i) Pyruvic acid + Coenzyme A  $\rightarrow$  Acetyl CoA + Formic acid

(ii) Glycine + Formate  $\rightarrow$  Serine  $\rightarrow$  Pyruvate

Literature concerning biosynthesis of other acids listed above are too scanty and need more attention before their actual role and metabolism in fungi may be understood. Some isolated references concerning them are mere records of their formation by a few fungi, and

are listed by Cochrane (1958) as well as Shibata *et al.* (1964).

## LIPID METABOLISM

Lipids generally include all those compounds which can be extracted from biological materials by organic fat solvents, like acetone, ether-alcohol mixtures etc. Such crude lipid mixtures extracted from natural sources are commonly referred to as "fats" or oils. Chemically speaking fats include the esters of glycerol with fatty acids. Complex lipids include (i) the phospholipids, in which one hydroxyl group of glycerol is esterified with phosphoric acid, and (ii) the glycolipid, in which glycerol forms a glycosidic bond with a sugar

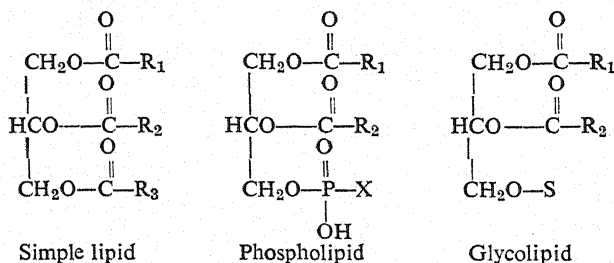


Fig. 6.1. Structure of some lipids.  $\text{R}_1\text{R}_2\text{R}_3$ =fatty acids, P=phosphate; S=sugar X=various, including choline, ethanolamine, serine, sphingosine etc.

Lipid constituents of fungal cells are known to vary both in quality and quantity in different fungal species. However, the fats, the fatty acids, the phospholipids, the sterols, the carotenoids, the triterpenes etc. are the general lipoidal materials; which are more significant in the fungal lipid metabolism.

### Break-down of Lipids

It is generally considered that the reactions leading to the break-down of carbohydrate substrates are the principal energy yielding processes. However, it should also be emphasized that degradation of lipoidal substances also provides a large amount of energy to the cell. In fact, degradation of a given quantity of fatty acid to carbon dioxide and water yields much more energy than what is obtained from the oxidation of the same amount of sugar, due to the low oxygen-content of the former.

Most of the fungi studied so far are able to utilize different fatty substances including true fats (triglycerides), phospholipids and fatty acids.

**Fats.** Fat-utilization is quite common among fungi, although these are considered as poor sources of carbon. Their utilization is catalyzed by the enzyme generally referred to as lipases. Lipase-activities have been reported in almost all the fungi, which have been investigated so far. However, the common method of lipase assay have so far employed simple esterases as the indicator of lipase-activity, despite the fact that all the esterases may not essentially hydrolyze fats. Zeller (1916) reported that an esterase isolated from *Lenzites sepiaria* failed to act upon fats. More realistic estimation of lipase activity among fungi, therefore, may be made by employing water-soluble synthetic substrates, as reported by Dingle and Solomons (1952) and Bier (1955). Available reports on fungal lipases, suggest that these enzymes are mainly intracellular, although appearing of late in the medium also (Yasuda *et al.* 1951 Crewther and Lennox, 1953; Stern *et al.* 1954). However, in some cases, both intra and extracellular lipases have been distinguished. For example, Fodor and Chari (1939) reported two types of lipases in *Aspergillus niger* and *Penicillium roqueforti*. *Mucor mucedo* also produces an extracellular lipase, which shows a correlation between its quantity and temperature of incubation. Also, more lipase activity is noticed, when this fungus is grown on a medium containing lipoidal materials (Stern *et al.* 1954). No definite trend has, however, been indicated regarding the lipase activity and pH of the medium.

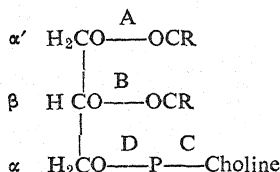
Action of lipase enzymes first, of all causes the hydrolysis of fats into glycerol and fatty acids. Glycerol is subsequently phosphorylated by ATP and oxidized to phosphoglyceraldehyde (PGAL) which may be further metabolised by the usual glycolytic and tricarboxylic acid sequence.

Fatty acid, on the other hand is degraded through an oxidative sequence of reactions, which to a great extent resembles the  $\beta$ -oxidation pathway known for animals, and differs from the latter only in the final steps. Details of the sequence have been described later.

**Phospholipids.** Phospholipids include all such lipoidal substances which contain phosphorus. The most common phospholipids are the glycerol phospholipids which are also some times referred as phosphoglycerides or phosphatides. In phosphoglycerides, one of the three alcohol groups of glycerol is esterified by a phosphate containing group instead of a fatty acid. This phosphorus containing group may be different in different kinds of phosphoglycerides.

Enzymes catalyzing the hydrolysis of phosphatides are designated as phospholipases, or specifically as lecithinase, cephalinase etc. acting upon the complex phosphoglycerides lecithins and cephalins res-

pectively. Lecithin and cephalins contain respectively the amino-alcohol choline and ethanolamine, in addition to a phosphate group. Obviously, these complex phosphatides possess several such bonds which are susceptible to hydrolysis by phospholipases, as is shown below for lecithins:



It has been observed that phospholipases from diverse sources, are able to cleave such a molecule from different points (Sumner and Somers, 1953) and according to the point of cleavage, the phospholipases have been distinguished into A, B, C and D types. Fragmentary reports of the occurrence of these enzymes indicate that phospholipase A is produced by *Aspergillus oryzae* (Contardi and Ercoli, 1935) and *Lycoperdon giganteum* (Francioli, 1935); phospholipase B by *Penicillium notatum* (Fairbairn, 1948); and phospholipase C by a species of *Aspergillus* (Akamatsu, 1923). Lecithinase D has been reported to be formed by the bacterium *Clostridium welchii*.

Such specific action of phospholipases results in the formation of partially degraded products. For an instance, the lecithin, after removal of one fatty acid residue by a phospholipase acting at A or B position is transformed into lysolecithin.

**Degradation of fatty acids.** All those monocarboxylic organic acids which are more soluble in organic solvents than in water, are included under fatty acids. Naturally occurring fatty acids have straight unbranched chains composed generally of even number of hydrocarbon residues. Fatty acids may have a saturated or unsaturated structure, but a double bond is seldom present between the carboxyl group and the ninth carbon atom removed. The fatty acids must undergo degradation before they are utilized by fungi.

Degradation of fatty acids by fungi is accomplished by an oxidative sequence of reactions, which is essentially an oxidation of the beta-carbon (2nd from the carboxyl group) of the fatty acid molecule. The overall pathway for fatty acid degradation in fungi seems to be essentially similar to the  $\beta$ -oxidation pathway elucidated for animal systems, although direct enzymatic evidences are still scanty.

In most eucaryotic cells the principal sites of fatty acid catabolism are the mitochondria, although among fungi this is yet to be demonstrated conclusively. In animal system, the whole pathway (Fig. 6.2)

has three main steps, viz. (a) addition of coenzyme A to the fatty acid molecule and activation of the latter, (b) oxidation of the beta-carbon,

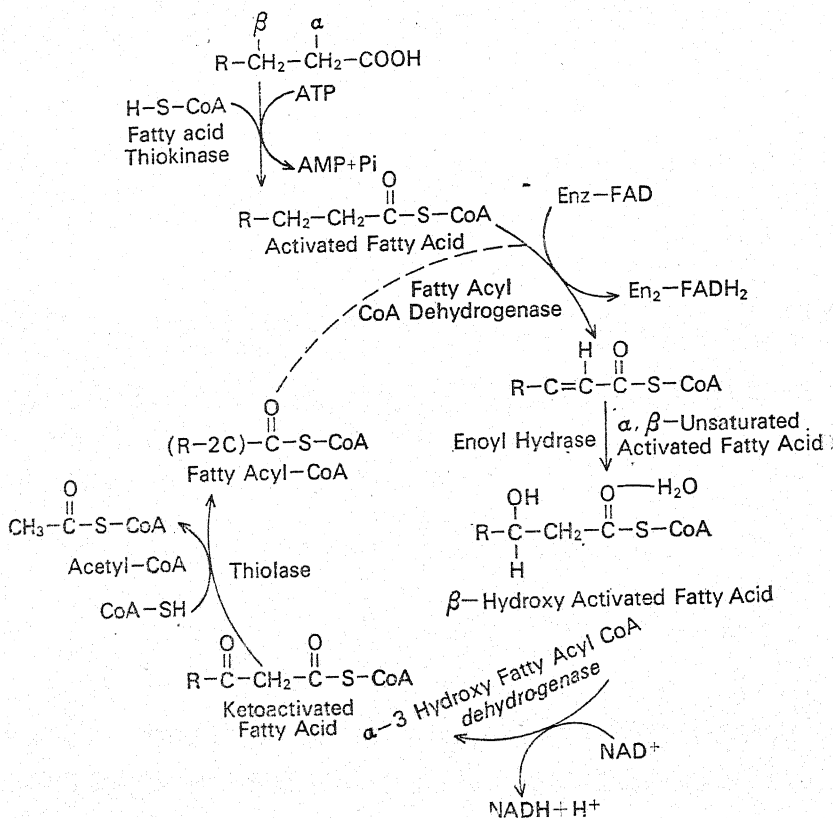


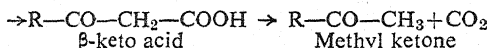
Fig. 6.2.  $\beta$ -oxidation of fatty acids as it occurs in animal tissues.

and (c) splitting of the acetyl CoA, resulting in removal of two carbon atoms from the fatty acid molecule. The resulting depleted fatty acid molecule, which has now a carbon chain shorter by two carbon atoms, repeats the sequence, which obviously is a spiral rather than a cycle. Thus the sequence goes on repeating till a 2-carbon fragment (in case of even numbered fatty acid) or a 3-carbon fragment viz. propionyl CoA (in case of an odd numbered fatty acid) is left out. The acetyl CoA is directly metabolized through TCA cycle, while propionyl CoA is first transformed into succinyl CoA and is then incorporated into the TCA cycle.

Among the fungi, evidences for the first and second steps are available, although far from sufficient. Regarding the final step, however,

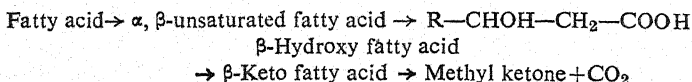


the evidences presently indicate that in fungi, only one carbon atom is removed (instead of two as in animals), resulting in the transformation of beta-keto acid into methyl ketone:



Presence of methyl ketones in fungal cheeses, in fungi contaminated edible oils, as well as fungi growing on different fatty acids *in vitro* has been amply demonstrated (Starkle, 1924; Stokoe, 1928; Coppock *et al.* 1928; Acklin, 1929; Thaler and Geist, 1939; Thaler and Stahlin, 1949; Thaler *et al.* 1949; Yamamoto, 1950; Mortimer and Johnson, 1952). Yet, it is presumed that complete oxidation upto the level of 2-carbon acetyl fragments is also possible among fungi, although direct evidence is lacking (Cochrane, 1958). Such assumptions are chiefly based on a few reports that some fungi produce acetoacetic acid during the oxidation of butyric acid (Coppock *et al.* 1928; Mukherjee, 1952), presumably as a result of combination of two acetyl fragments. No direct evidence is, however, available to suggest the occurrence of the complete  $\beta$ -oxidation pathway in fungi, although some indirect evidences, like incorporation of fatty acid carbon residue into the side chain of penicillin (Thorn and Johnson, 1950; Mortimer and Johnson, 1952), and presence of fatty acid oxidase and lipoxidase enzyme systems in certain fungi like *Aspergillus* spp. and *Penicillium glaucum* (Mukherjee, 1951) do indicate that possibility.

Thaler and his associates (1949) studied the utilization of fatty acids and their derivatives by fungi and concluded that fungi degrade these substances by the following sequence of reactions producing methyl ketone:



Production of methyl ketone has been recorded during fungal degradation of a variety of fatty acids, including butyric acid, octanoic acid, dodecanoic acid etc. Also, the  $\beta$ -keto acid decarboxylases are known to occur in fungi (Karrer and Haab, 1948).

Thus, our present limited knowledge on this aspect, as well as conjectural evidences indicate that fatty acid oxidation by fungi is accomplished very much by the same sequence as is known for animal systems, except for the final step of the pathway, which leads to the formation of methyl ketones by fungi, probably as a detoxification mechanism against the fatty acids. It is more than obvious

that much remains to be learnt about this aspect of fungal metabolism.

### Lipid Synthesis

Fungal mycelium store fatty substances, which may in some cases account upto 50% of the mycelial dry-weight. Fat synthesized by fungi, however, comprise mainly neutral fats and free fatty acids.

Kordes (1923) studied the fat content of eighteen different moulds and recorded fat-accumulation in spores and old hyphae of *Mucor* spp., *Rhizopus nigricans*, *Absidia cylindrospora*, *Aspergillus* spp. *Claviceps purpurea*, *Pleospora herbarum*, *Sclerotinia tuberosa* and *Daedalea quercina*. Fat-production by *Aspergilli* and *Penicillia* has been studied consistently (Pruess *et al.* 1934; Ward *et al.* 1935; Woodbine *et al.* 1951) and they appear to be prolific producers of fungal fats. However, species of *Endomyces*, *Torula*, *Geotrichum* etc. are also good sources of mycological fat. Very high yield of fat was recorded in *Mucor circinelloides* (54.2%), *Fusarium bulbigenum* (45.6%) *Zygorhynchus moelleri* (39.9%) and *Endomyces vernalis* (31.1%) by Bernhauer *et al.* (1948). Fungi show variation in their fat producing abilities not only at specific level but even at strainal or clonal levels. Nutritional and environmental factors also influence the amount of fat synthesized by fungi. Although fat production may occur on any carbohydrate as the carbon source, but certain sugars support higher fat-production in specific fungi. Glucose and xylose were superior for *Aspergillus nidulans*, *Penicillium spinulosum*, and *P. javanicum* (Gad and Walker 1954), while maltose, sucrose, arabinose, galactose, lactose and starch were inferior C-sources for fat-production. Concentration of the carbohydrate and more particularly the ratio of carbon and nitrogen sources also play decisive role in fat-production. Generally, increase in the level of carbohydrate results in enhanced fat-production, except in those fungi, where excessive glucose inhibits the growth. In contrast to C-source, nitrogen source is required only in suboptimal quantity to support maximum fat production. Garrido and Walker (1956) found that out of 5 different N-sources, ammonium nitrate supported maximum fat-synthesis in all the three fungi, they studied. Similarly, certain metallic ions as well as phosphate ions (Kleinzeller, 1948) also have their effects upon fat accumulation. Effects of some physical factors like temperature and pH of the medium on fat synthesis have also been studied. Available reports on this aspect indicate that the temperature optima for fat-production

and growth in general are almost identical (Kleinzeller, 1949; Litvinova and Raevskaya, 1952; Gad and Walker, 1954; Pupowa and Putschkova, 1967; Mehrotra and Krishna Nand, 1970). However, there are several reports to suggest that the quality of the fat synthesized at different temperatures may vary (Pearson and Raper, 1927; Terroine *et al.* 1927; Bass and Hospodka, 1952; Singh and Walker, 1956).

In contrast to temperature, pH of the medium has definite influence on the amount of fat produced. In fact, different fungal species exhibit different pH-range as well as different pH-optima (Table 6.1).

TABLE 6.1  
SHOWING pH-OPTIMA OF DIFFERENT FUNGI FOR FAT PRODUCTION

Fungi	pH-optima/ pH-range	Author/s
1. <i>Penicillium javanicum</i>	4.5-5.5	Cioffi & Varetto (1951)
2. <i>Rhodotorula gracilis</i>	3.0-6.5	Steinberg & Ordal (1954)
3. <i>Torulopsis lipofera</i>	5.5-6.0	Kleinzeller (1948)
4. <i>Hansenula anomala</i>	6.5	Brock (1956)
5. <i>Mucor recurvus</i>	6.0	Krishna Nand & Mehrotra (1970)
6. <i>Phycomyces blakesleeana</i>	6.5	Do
7. <i>Mucor mucedo</i>	7.0	Bline & Bojee (1942)
8. <i>Mucor circinelloides</i>	8.5	Krishna Nand & Mehrotra (1970)
9. <i>M. peacockensis</i>	"	Do
10. <i>Aspergillus allahabadii</i>	"	Do
11. <i>A. indicus</i>	"	Do
12. <i>Penicillium oxalicum</i>	"	Do

It may be noted from the Table 6.1 that some fungi require acidic pH while others need alkaline pH of the medium for optimum fat-production. However, there are reports which suggest that lower-pH of the medium inhibits fat-production (Foster, 1949; Smedley-Maclean and Hoffert, 1923). Smedley-Maclean (1922) concluded that at low pH, lipid synthesis is inhibited and the assimilated glucose is transformed into complex carbohydrates; while at higher pH conversion of glucose into lipoidal materials is favoured. In many of the studies, pH-optima for growth and fat-production were distinctly different.

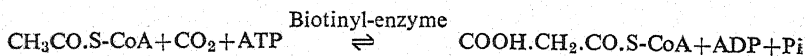
Fats are the reserve substances and are formed intracellularly possi-

bly in association with cytoplasmic particles (Steiner and Heinemann, 1954). The two components required for synthesis of fatty substances are (i) glycerol and (ii) fatty acids, both of which are known to be synthesized by fungi. Glycerol may be derived from dihydroxyacetone phosphate, which is produced during glycolysis, the sequence of reactions involved has been presented in chapter VII. The biosynthesis of fatty acids is described below:

### Biosynthesis of Fatty Acids

In most of the earlier studies on fatty acid synthesis by fungi, suitable precursors were employed, which indicated that the fatty acid carbon-skeleton is derived from some 2-carbon compounds. Studies with a mutant strain of *Neurospora crassa* requiring exogenous acetate indicated that the carbon of fatty acids required by this fungus was entirely derived from exogenously provided acetate. Isotopic studies in several other fungi, including *Phycomyces blakesleeana* (Bernhard, 1948), *Fusarium lini* (Coleman *et al.* 1952) and *Ustilago zaeae* (Fahraeus and Lindeberg, 1953) have also furnished data which establish the role of acetate in the synthesis of fatty acids. It is now well recognised that the carbon atoms required for fatty acid synthesis are contributed, two at a time by acetyl-CoA molecules. The synthesis of fatty acid molecules from acetyl-CoA, thus involves (i) formation of carbon to carbon linkage between acetyl radicals, and (ii) reduction of ketone groups to the hydrocarbon stage. It is obvious that both these steps require energy, and the first step which is essentially a condensation step is more difficult to accomplish. The reductive step is supported by NADPH.

During the condensation step, the methyl group of an acetyl CoA is not so reactive as to combine with another acetyl unit, particularly under biological conditions. It was observed by Klein (1957) that  $\text{CO}_2$  exerted stimulatory influence upon lipid synthesis in cell-free extract from yeast. This was ascribed to the formation of malonyl-CoA (Den and Klein, 1961; Lynen, 1961) due to combination of  $\text{CO}_2$  with an acetyl-CoA molecule by the following reaction:



Production of malonyl-CoA furnishes a more reactive radical, which may unite with an acetyl CoA or acyl CoA of a higher fatty acid to form the carbon-chain. Once the carbon skeleton is ready, there follows the reductive phase, during which reduction of the ketone group into hydrocarbon form is accomplished, and a fatty acid molecule is thus synthesized. The entire process occurs in seven different

steps in form of a cycle (Lynen, 1961). Enzymes catalyzing the different steps of the cycle have not been isolated from fungi, although from *Escherichia coli* five of six enzymes involved in the seven steps of the cycle have been isolated. In yeast, Lynen (1961) has suggested that six enzymes remain arranged round a functional sulphydryl group, which firmly binds the intermediates of fatty acid synthesis in close proximities to the active sites of the component enzymes.

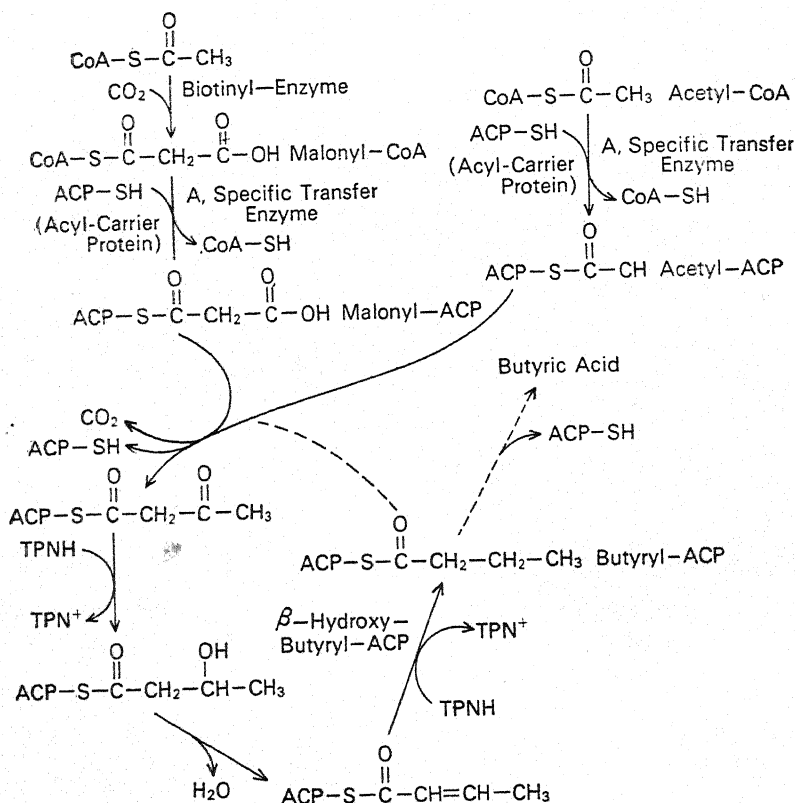


Fig. 6.3. Bio-synthesis of fatty acids.

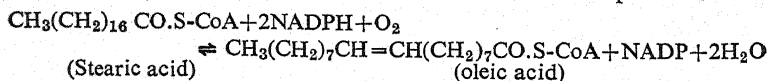
The synthesis of malonyl-CoA from a molecule of acetyl-CoA and that of  $\text{CO}_2$  (actually as  $\text{HCO}_3^-$ ) is a complex process and involves at least two steps:

- (i)  $\text{CO}_2 + \text{ATP} + \text{biotinyl-enzyme} \rightarrow \text{Carboxybiotinyl-enzyme} + \text{ADP} + \text{P}_i$
- (ii)  $\text{Carboxybiotinyl-enzyme} + \text{CH}_3\text{CO.S-CoA} \rightarrow \text{biotinyl enzyme} + \text{COOH.CH}_2\text{CO.S-CoA}$

The condensation stage is yet to be completely understood. In many

cases, specific transfer enzymes transfer the malonyl and acetyl radicals from coenzyme A to a small protein called the acyl-carrier-protein (ACP); which has a prosthetic group with structure very much identical to that of CoA. This step leads to the formation of malonyl-ACP and Acetyl-ACP. Next follows the condensation of the malonyl- and acetyl-ACPS, during which acetyl radical is transferred to the malonyl group while the carboxyl group of the latter is lost as  $\text{CO}_2$ . The resulting compound is thus acetoacetyl-ACP. It is obvious, therefore, that  $\text{CO}_2$  plays essentially the role of a catalyst. Next few steps bring about reduction of the ketone groups and ultimately butyryl-ACP is formed. Then there are two possibilities. Either the butyric acid is released from ACP, which seldom occurs, or the butyryl-ACP may react with another molecule of malonyl-ACP, so that the cycle may begin a fresh turn. In the second event, the chain-length goes on increasing by two carbon atoms with each turn of the cycle, and it is not yet clear what actually stops the cycle to determinate the chain-length of the fatty acid. But it does stop when the chain-length is around  $\text{C}_{16}$  to  $\text{C}_{18}$ . Palmitic acid [ $\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$ ] is the principal product of yeast, together with a little of myristic [ $\text{CH}_3(\text{CH}_2)_{12}\text{COOH}$ ] and stearic [ $\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$ ] acids.

Nutritional data from fatty acid-requiring mutants of *Neurospora crassa* (Lein *et al.* 1953), as well as analysis of the mycelial content of *Aspergillus nidulans* (Singh and Walker, 1956) indicated that separate routes operated for the biosynthesis of saturated and unsaturated fatty acids in these fungi, and there appeared to be little chance for a direct derivation of unsaturated acids from saturated ones by dehydrogenation. But in yeasts unsaturated acids are possibly synthesized from saturated ones by an aerobic particulate system, according to the following reactions, in which NADPH also takes part:



Another example of such transformation is also recorded in a yeast, *viz.* *Candida* (Meyer and Bloch, 1963), where oleic acid is desaturated into linoleic acid possessing two double bonds [ $\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$ ].

Biosynthesis of complex lipids by fungi is but little understood and further work on this aspect is awaited.

## METABOLISM OF STEROIDS

Synthesis of sterols in fungi appear to proceed side by side with fat

synthesis. Most fungi accumulate small amount of sterols, among which ergosterol seems to be the most common. Other important steroidal products of fungi are ergot-alkaloids from *Claviceps pur-*

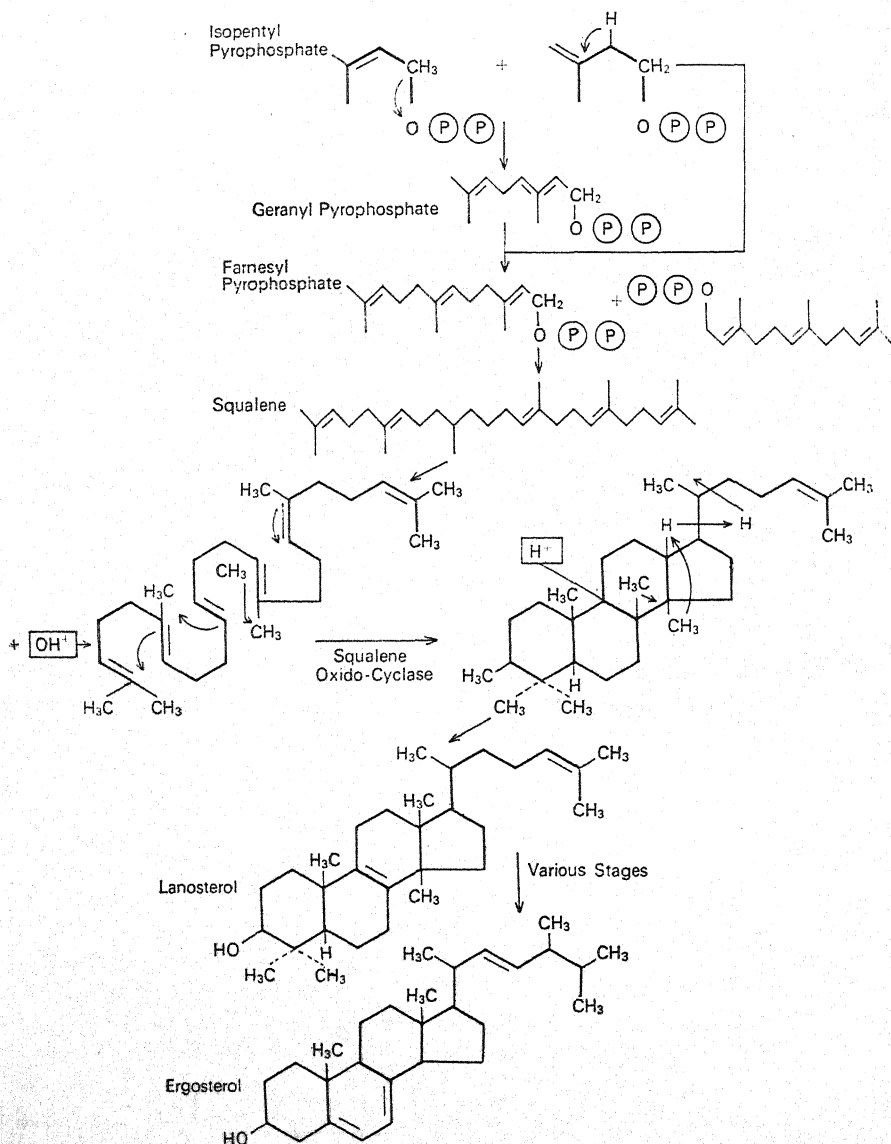


Fig. 6.4. Probable pathway of Ergosterol-synthesis.

*purea*, hallucinogenic steroids of *Psilocybe* sp., and gibberellins produced by *Fusarium moniliforme*. The last two kinds have been discussed in Chapter XVI. Only ergosterol is described here.

Ergosterol, may occur in pure form in a particulate fraction as in yeast (Klein and Booher, 1956), in form of crystals, as in *Neurospora* (Tsuda and Tatum, 1961) or as palmitate compound as in *Penicillium chrysogenum* (Miyazaki *et al.* 1962). The biosynthesis of ergosterol is shown in Fig. 6.4 which shows that squalene lanosterol, farnesyl pyrophosphate etc. are the precursors of ergosterol. Synthesis of squalene from mevalonate via farnesyl pyrophosphate has been observed in yeast (Schwerk and Alexander, 1958; Henning *et al.* 1959). Evidences from other organisms indicate that lanosterol is also a precursor of ergosterol, but the enzyme squalene-oxidocyclase has not been isolated from fungi.

Several other steroids and their transformation products are known among fungi, some of which are shown in Fig. 6.5 alongwith the name of fungi which have been reported to cause those transformations.

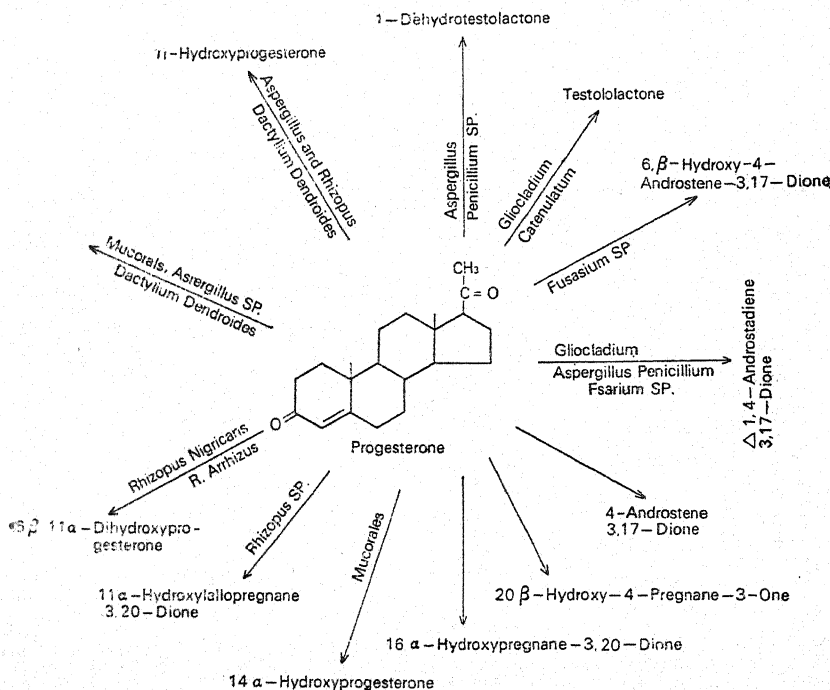


Fig. 6.5. Some Steroids produced by Fungi.



## METABOLISM OF AROMATIC COMPOUNDS

Fungi are distinctly different from bacteria in their ability to produce a variety of aromatic metabolites from carbohydrate substrate. Such cyclic compounds are synthesized as a result of primary and secondary metabolism both. A number of such products have been listed in Chapter XVI. The present discussion will include the utilization of those aromatics which are produced by the fungi during their primary metabolic phase and are later utilized as source of carbon and energy.

Many fungi possess specialized metabolic pathways through which they utilize aromatic substances and convert them to aliphatic cellular components. One of such pathways for dissimilation of aromatic and hydroaromatic compounds, although yet to be fully elucidated in fungi, is  $\beta$ -ketoadipate pathway. The pathway is widely distributed among fungi and provide them a mechanism for utilization of a variety of primary substances.

### $\beta$ -ketoadipate Pathway

Little was known of the mechanism of biological conversion of benzenoid compounds into aliphatic products, before this pathway was elucidated. Investigation in this direction began with an attempt by Charles Evans (1947) to study the accumulated products in the culture media containing benzoic acid; *p*-hydroxybenzoic acid and phenol, due to growth and metabolism of an aerobic bacterium, which was later identified as *Acinetobacter calcoaceticus*. He identified diphenols, catechol and protocatechuic acid in the culture-filtrate, besides the Keto acids. Kilby (1948) isolated a keto acid from such culture-filtrate and identified it to be  $\beta$ -ketoadipic acid and demonstrated that the cells which may grow on aromatic substrates dissimilate this acid. Most other evidences as well as clues for elucidation of this pathway have been obtained from studies on other aerobic bacteria and fungi seem to have contributed very little. It is perhaps on this account that the pathway is not yet completely elucidated in fungi. Stanier and Ornston (1973) have presented a detailed review of the history, chemistry genetics and various other aspects of this pathway, and here only a brief treatment of the sequence as known in fungi will suffice.

The initial part of the  $\beta$ -ketoadipate pathway is concerned with the conversion of the various primary aromatic substrates into protocatechuic acid or into catechol. These conversions occur through two independent sequences, which are shown in Fig. 6.6 and Fig. 6.7.

Formation of protocatechuate and catechol marks the sites of entry to the two parallel and convergent branches of the  $\beta$ -ketoadipate pathway.

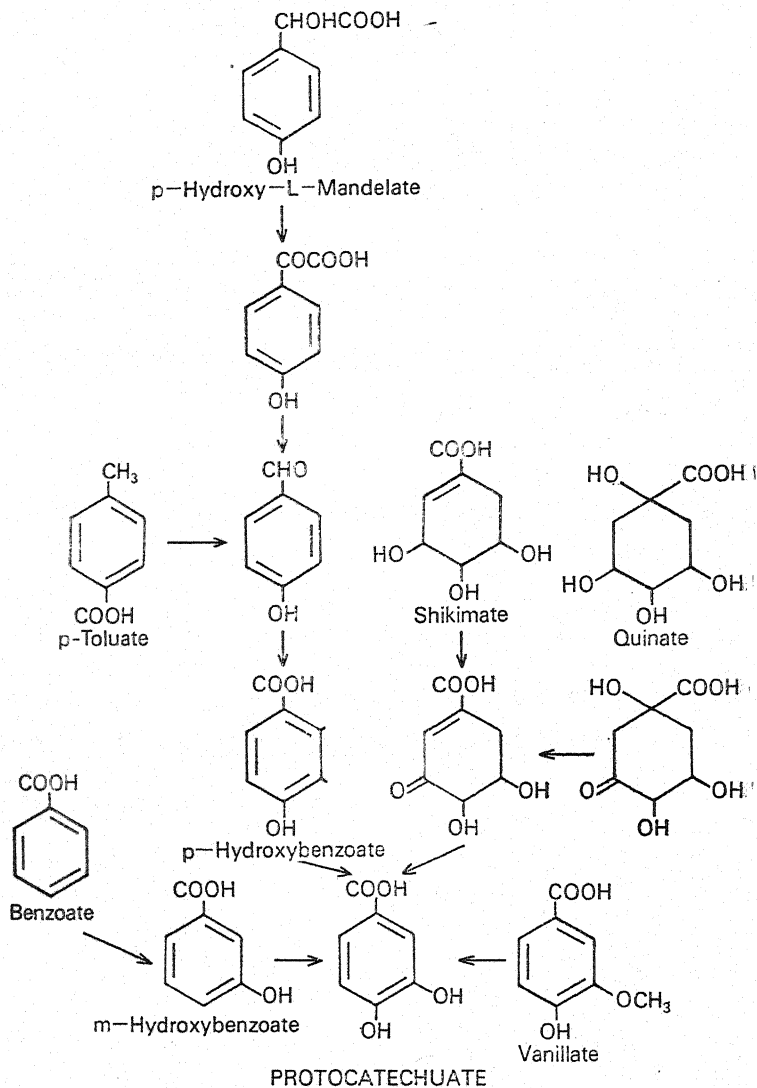


Fig. 6.6. Initial sequence (A) of reactions of  $\beta$ -ketoadipate pathway.

pate pathway (Fig. 6.8). These diphenols undergo the various steps of the pathway and are ultimately converted into succinate and acetyl-CoA, which are the end-products of  $\beta$ -ketoadipate pathway. The path-

way operating in fungi shows an overall similarity to the one known in bacteria, particularly with respect to the catechol branch. The only

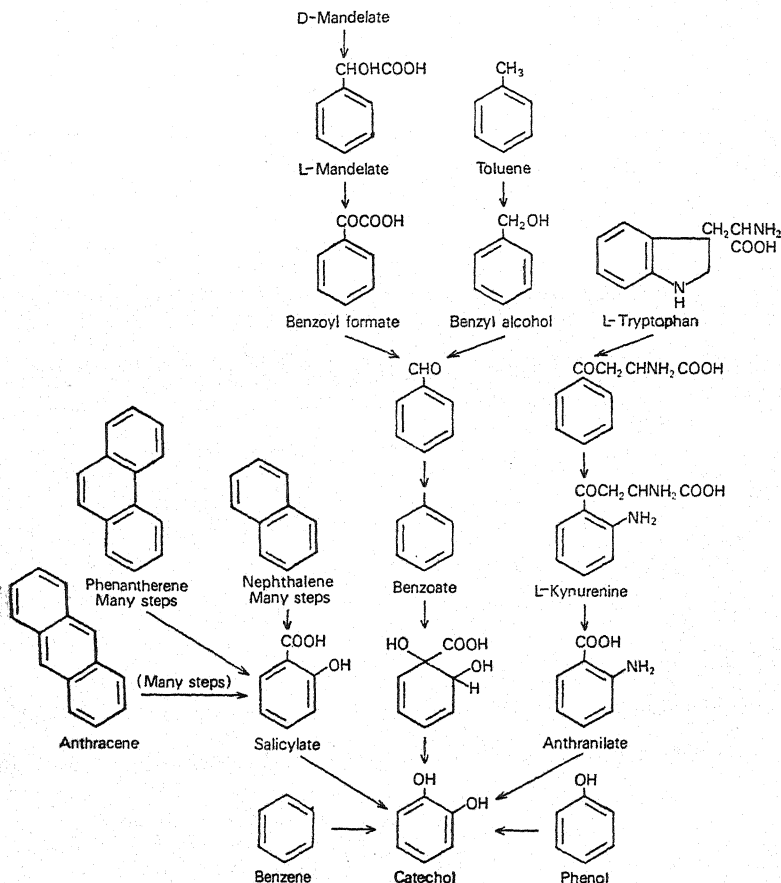


Fig. 6.7. Initial sequence (B) of  $\beta$ -ketoadipate pathway.

divergence recorded in fungi is with respect to the reactions of the protocatechuate branch. This deviation was first shown in *Neurospora* by Gross *et. al.* (1956). The lactonization of  $\beta$ -carboxy, *cis*, cismuconic acid yields the  $\beta$ -lactone in fungi which is subsequently converted into  $\beta$ -ketoadipic acid through a sequence of reactions (Fig. 6.9), which is yet to be fully elucidated. In bacteria, on the other hand, the lactonization of  $\beta$ -carboxy-*cis*, *cis*-muconate yields  $\gamma$ -carboxymuconolactone, whose conversion into  $\beta$ -ketoadipate takes place through slightly different and simpler route. The slightly different  $\beta$ -lactone

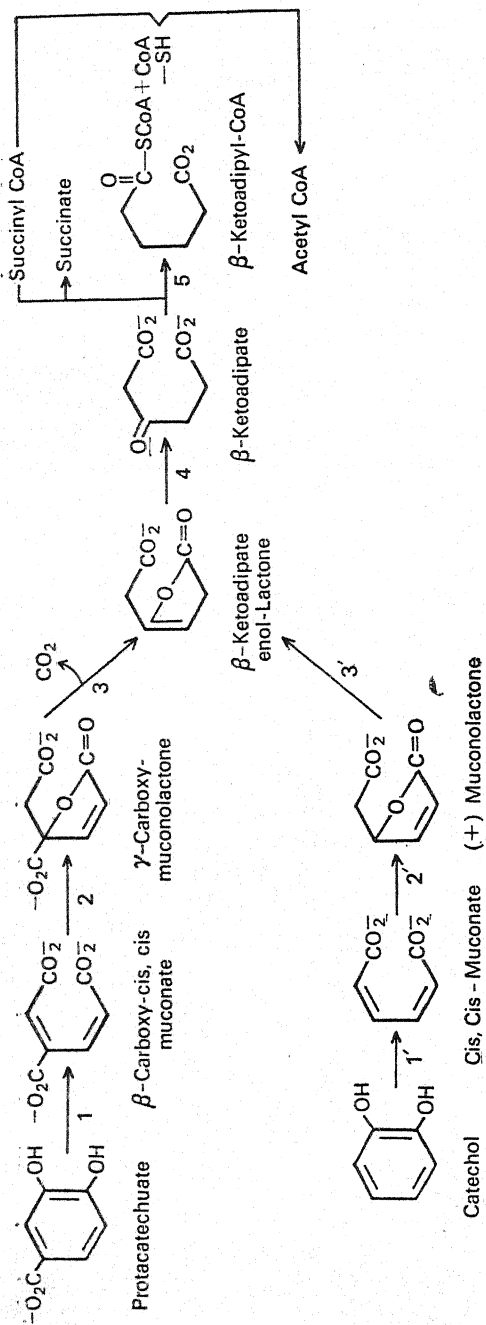


Fig. 6.8. The central reactions of the  $\beta$ -ketoadipate pathway in bacteria.

route has now been recorded in several fungi and it appears to be the characteristic feature of fungi. However, the sequence is yet to be completely elucidated and many of the intermediates are still doubtful, which have been shown within parentheses.

It may be noted that both during the initial as well as the central reactions of this pathway, most of the oxidative steps are mediated

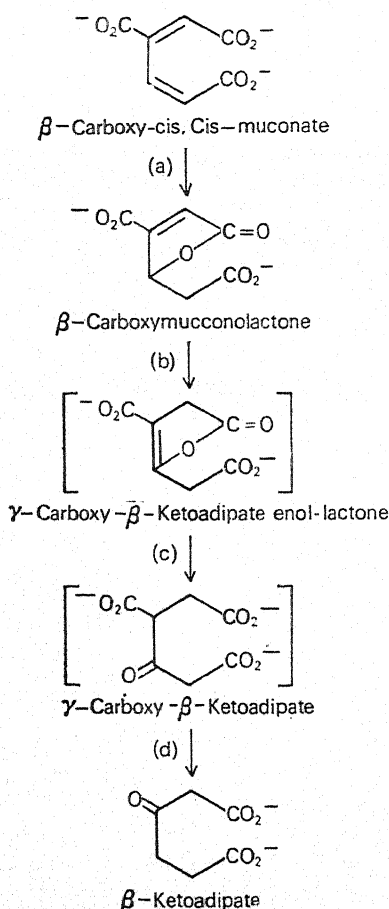


Fig. 6.9. Probable route for fungal conversion 2  $\beta$ -carboxy *cis,cis* muconate to  $\beta$ -ketoadipate.

by the enzymes oxygenases, and not dehydrogenases (Hiyaishi, 1966). Therefore, this pathway is aerobic in a very strict sense, and stops functioning in absence of molecular oxygen. They can not function even when some substitute electron acceptor like nitrate is present in place of oxygen. Another effect of operation of oxygenases enzymes in this pathway is the complete omission of the respiratory electron transport-system, because oxygen is reduced at the substrate level itself. It is obvious that omission of terminal oxidation leads to omission of oxidative phosphorylation also and, therefore, no ATP molecules are synthesized as a direct consequence of this pathway. Of course both the end-products of this pathway, *viz.* acetyl-CoA and succinate may enter the TCA cycle and their degradation may yield ATP molecules as indirect products of the  $\beta$ -ketoadipate pathway.

The pathway seems to be quite interesting and complex, but at the same time quite specialised. Further work to establish its uncertain steps in fungi as well as its

complex enzymology requires immediate attention, particularly the latter aspect which is known to any considerable extent only among bacteria and that too only in *Pseudomonas* spp.

## RESPIRATION—I

## GLYCOLYSIS

All energy yielding reactions in a living cell, which cause the conversion of chemical bond energy of organic molecules into metabolically usable energy comprise respiration. Removal of the bond energy during respiratory reactions is generally accomplished by withdrawal of hydrogen from the respiratory substrates. In other words living cells know only one kind of fuel, *i.e.* hydrogen. Majority of the reactions of respiration may be represented as shown in Fig. 7.1.

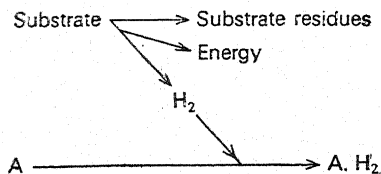


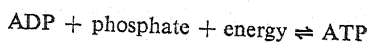
Fig. 7.1.

Where  $A$  acting as the hydrogen-acceptor, helps in the removal of hydrogen from the substrate. Various substances may act as hydrogen-acceptor. If the ultimate hydrogen acceptor is oxygen the respiration is said to be aerobic one, while if a substance other than oxygen serves as the final hydrogen-acceptor, the respiration is anaerobic. However, dehydrogenation in both the forms of respiration requires the catalytic activities of some specific enzymes, called the respiratory enzymes, which drive the processes in appropriate direction.

Although any cell constituent containing breakable carbon bond like carbohydrates, fats, proteins, their derivatives etc. may serve as the substrate for respiration, the compounds of the first two categories are generally the principal substrates for respiration. This, however, does not mean that the structural components of cells are spared from respiratory degradation. The cell components are also gradually

degraded and are continuously replaced by newer counterparts. This obviously means that the destructive energy metabolism (catabolism) and the constructive synthetic metabolism (anabolism) go together simultaneously and maintain a sort of balance between them.

Like the hydrogen, the energy released from the substrate is also accepted by certain specific cell components *viz.*, adenosine phosphates. A common energy trapping reaction in a living systems functions as follows:



Energy liberated from respiratory substrate is incorporated into ADP and phosphate to form ATP. The ATP not only stores the energy, as shown above, but also acts as energy carrier and thus supplies energy when and where it is needed. When ATP serves as the energy donor, the equation shown above is functional towards the left.

Thus respiration involves three correlated phases: (i) dehydrogenation of the substrate with the help of a hydrogen acceptor; generally nicotinamide adenine dinucleotide, oxygen, etc. serve as hydrogen acceptors, (ii) substrate degradation by cleavage of the carbon to carbon bonds; the breakdown may be complete, *i.e.* upto 1-carbon level, or may be incomplete upto 2 or 3 carbon level, and (iii) energy transfer, which involves the incorporation of energy, liberated from the respiratory substrate, into ATP, which thus comprise the main product of respiration. The three aspects of respiration are schematised in Fig. 7.2.

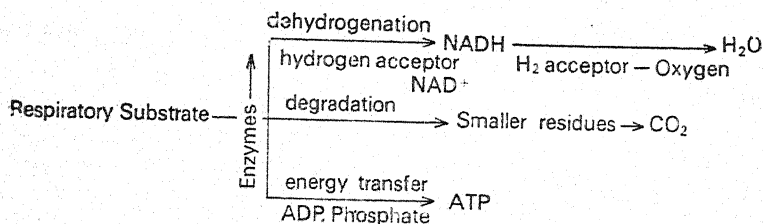


Fig. 7.2.

Probes into the physiology and biochemistry of fungal respiration, during the last century—since Pasteur (1860) published his impressive, "Memoir on Alcoholic Fermentation" and established the role of yeasts in the process—has shown that the fungi generally hold well the Kluverian concept of unity of biochemistry. Demonstration of glycolysis in yeasts (Buchner, 1897), and subsequent elucidation of the Embden-Meyerhof (EM) pathway in yeasts and later in fila-

mentous fungi (Lynen and Hoffmann-Walbeck, 1948); recognition of yet another glycolytic sequence *viz.* hexose monophosphate (HMP) pathway in yeast (cf. Dickens, 1958) and almost simultaneously in filamentous fungi indicate the extent to which fungi, and particularly the yeasts have been exploited for the understanding of the respiratory reactions. However, recent advances in our knowledge of fungal respiration has been comparatively slower, generally ascribed to some technical lacunae. The two important limitations in this regard are, (i) limited availability of experimental samples of fungi of satisfactory homogeneity and reproducibility; and (ii) high rate of endogenous of auto-respiration in fungi, which interferes with interpretation of their respiratory efficiency on exogenous substrates, and also because it makes the inhibitor-study difficult.

It is now generally accepted that Embden-Meyerhof (EM) and hexose monophosphate (HMP) pathways of glycolysis are operative in many fungi, and that the overall respiratory mechanism is generally similar to that obtaining in other organisms involving the tricarboxylic acid (TCA) cycle as well as the terminal oxidation with the help of electron transport or the respiratory chain.

However, as indicated by Foster (1958), fungi show immense possibilities of new metabolic pathways, and existence of a non-phosphorylative catabolic sequence among these organisms cannot be entirely ruled out, particularly in view of the recent discoveries of three such pathways in bacteria, which do not involve phosphorylated sugars. (cf. Hollmann, 1964). There have been a few reports of glucose oxidation without prior phosphorylation (see Chapter V) in some fungi but role of such oxidation in respiration is not clear.

### **Phosphorylative Carbon Catabolism**

Although fungi utilize various types of complex carbon compounds, the energy yielding reactions of respiration operate through the monomeric forms only. The monosaccharides like glucose, fructose and galactose may be utilized directly as respiratory substrates, while the polysaccharides (as well as the fats and proteins) are required to be degraded into their monomeric forms before they could enter the respiratory pathway. Fungi accomplish this initial degradation of such polymers with the help of specific extracellular enzymes, at or near the cell-membrane.

The overall catabolic sequence of respiration involves the following three main stages:

- I. Glycolysis, involving phosphorylation of the suitable hexose: unit generally glucose, and its subsequent degradation into 3-or 2



carbon compounds (pyruvic or lactic acid) via one or the other glycolytic pathways. This obviously means that conversion of a carbohydrate (except glucose) into suitable hexose unit must proceed its entry into the glycolytic sequence.

II. Tricarboxylic acid cycle, serving as a route for pyruvate utilization, during which the pyruvic acid is completely degraded to 1-carbon compound ( $\text{CO}_2$ ). A multitude of intermediates of this cycle serve as the carbon moieties for the synthesis of cell constituents. The energy liberated during this sequence is available to the cell in two useful forms, the reduced coenzymes NADH and  $\text{FADH}_2$  as well as ATP. However, pyruvic acid cannot enter the TCA cycle directly and hence its prior oxidative decarboxylation into acetyl-CoA is essential.

III. Terminal oxidation and electron transport, which involves the reoxidation of the reduced coenzymes by transfer of electrons through a series of enzymes called the respiratory chain, and finally to oxygen, producing water. This transport of electrons down the respiratory chain makes available free energy, which is utilized in the production of ATP, through oxidative phosphorylation.

### Glycolysis

Glycolytic reactions in fungi serve essentially the same functions as in other organisms and furnish metabolically usable energy, and various building materials for the biosynthesis of cellular constituents. As mentioned earlier, this phase of respiration accomplishes physiological degradation of glucose to pyruvic or lactic acids. The sequence of reactions begins with glucose, or with the storage carbohydrate glycogen and ends with the production of pyruvate or lactate. Other hexose sugars, like fructose and galactose may also enter the glycolytic reactions, but in every case the primary step of glycolysis involves the phosphorylation of the sugar. Glucose is phosphorylated to glucose-6-phosphate by the removal of the terminal phosphate group of ATP and its subsequent attachment to the 6th carbon atom of glucose by a sugar phosphate-ester linkage. The bond energy required for this linkage is more than compensated by the energy liberated from the splitting of the pyrophosphate bond of the ATP. Such direct phosphorylation of glucose is catalyzed by the enzyme glucokinase which also requires  $\text{Mg}^{2+}$  as a co-factor of ATP. Some other sugars, like fructose and mannose may also be phosphorylated in similar fashion under the influence of the respective hexokinases, but phosphorylation of galactose follows a different route as shown in Fig. 7.3.

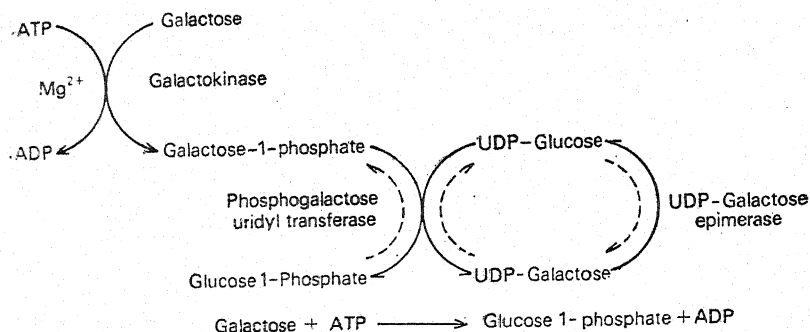


Fig. 7.3. Phosphorylation of galactose.

Different from other hexokinases, the galactokinase influences the phosphorylation of the 1st carbon atom of the sugar instead of carbon-6, and produces galactose-1 phosphate, in the presence of ATP. Subsequently, this is converted to glucose-1-phosphate under the influence of two different enzymes, viz. phosphogalactose uridyl transferase and UDP-galactose 4-epimerase.

Formation of hexose phosphate from glycogen is achieved by the enzyme phosphorylase which produces glucose-1-phosphate without the participation of ATP. Obviously, the splitting of the glycosidic bonds of glycogen provides the required energy, and inorganic phosphate is utilized for phosphorylation.

The various hexose phosphates, like glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate and mannose 6-phosphate are all interconvertible under the influence of enzymes like phosphoglucosmutase, phosphohexose isomerase etc. Interconversion of these phosphorylated hexoses are represented in Fig. 7.4.

Once the hexose 6-phosphate has been formed, further reactions may proceed in two different sequences or pathways viz. (i) the hexose diphosphate pathway, in which the break-down of the sugar is preceded by its further phosphorylation forming a hexose diphosphate; and (ii) the hexose monophosphate pathway, in which the actual break-down of the sugar is initiated at the monophosphate level itself and therefore, no further phosphorylation is required. The pathways differ in other details as well, which will be emphasized at appropriate places.

### Hexose Diphosphate Pathway: E.M. Pathway

The only well known pathway operating through the utilization of a doubly phosphorylated hexose is the Embden-Meyerhof (EM)

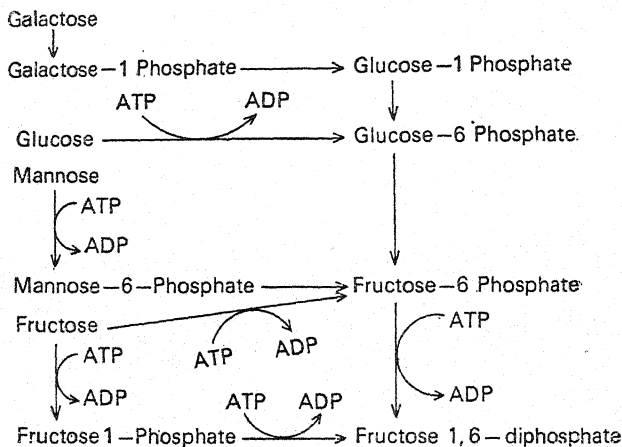


Fig. 7.4. Interconversion of hexoses.

pathway. This sequence of reactions, although not universal, occurs widely among the microorganisms. EM pathway is the most widespread one among fungi. Occurrence and role of different pathways among fungi is discussed later.

The chief characteristic of the EM pathway is a second phosphorylation of the sugar before its break-down sets in. For this the glucose 6-phosphate (which is the bifurcation-point of the hexose mono- and diphosphate pathways) is converted into fructose 6-phosphate under the influence of phosphohexose isomerase, which is subsequently subjected to phosphorylation at its other end. The enzyme catalyzing this reaction, leading to the formation of fructose 1, 6-diphosphate, is phosphofructokinase which is specific to the EM pathway.

The actual degradation of the hexose molecule sets herein, as the enzyme aldolase causes the splitting of fructose 1, 6-diphosphate into two triose phosphates, viz. glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The splitting of the fructose 1, 6-diphosphate is achieved in two to three freely reversible steps, during which intermediate products are formed. First of all an enzyme bound intermediate, the schiff's base is formed as the E-amino group of a lysine residue of the aldolase reacts with the ketone group of the fructose diphosphate. This enzyme-fructose complex gives rise to a glyceraldehyde 3-phosphate molecule and a triosephosphate-enzyme

complex, which ultimately yields a dihydroxyacetone phosphate. The mechanism is shown in Fig. 7.5.

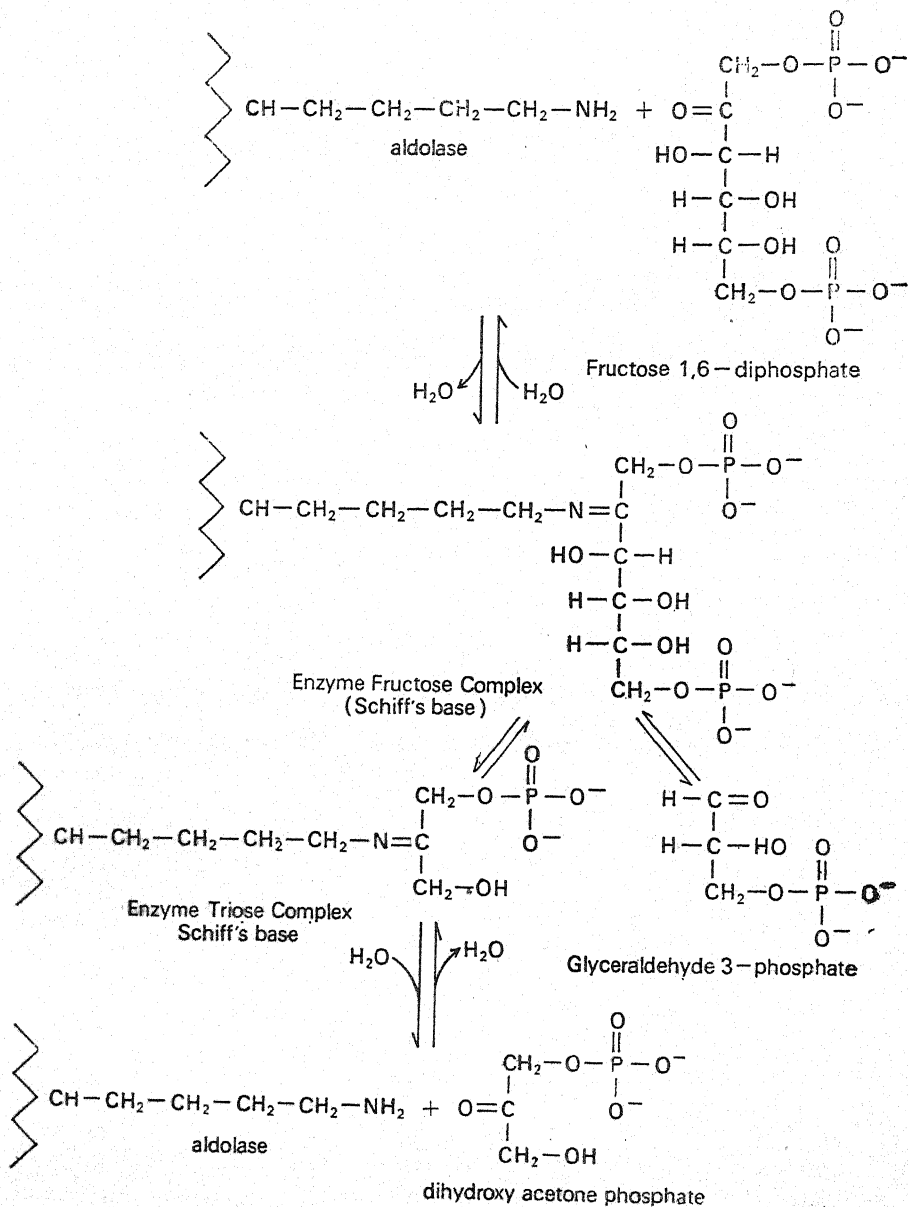


Fig. 7.5. Cleavage of fructose 1, 6-diphosphate catalyzed by aldolase.

The two triose phosphates are inter-convertible, but due to the oxidation of glyceraldehyde-3 phosphate into 1, 3-diphosphoglyceric acid by an enzyme triose phosphate dehydrogenase, the net transformation proceeds from dihydroxyacetone phosphate to glyceraldehyde 3-phosphate under the influence of isomerase. However, under certain conditions, e.g., during fermentation by some yeasts, dihydroxyacetone phosphate is reduced to form glycerol and other polyhydric alcohols like arabitol, erythritol and mannitol, *Saccharomyces cerevisiae* under alkaline conditions transforms dihydroxyacetone phosphate into glycerol 3-phosphate, which is dephosphorylated to glycerol. The sequence is schematized in Fig. 7.6.

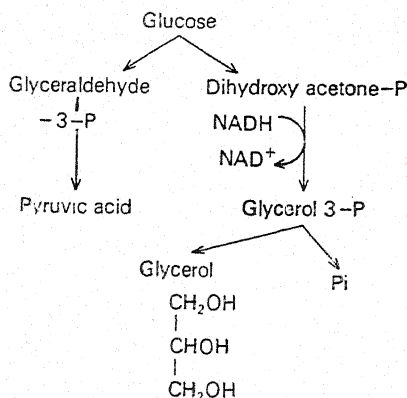


Fig. 7.6. Formation of glycerol.

It may be noted that this also marks the point of entry of glycerol into the glycolytic sequence. When the above reactions proceed in the opposite direction they provide for conversion of glycerol into dihydroxyacetone phosphate, which then participates in further steps of glycolysis.

The next few steps of the EM pathway (Fig. 7.7) are apparently more useful for the cell, because all the ATP producing reactions are confined to them. It may be emphasized that the steps described till now do not yield any useful energy, rather two high energy rich phosphate bonds per hexose molecule have been utilized.

The oxidation of glyceraldehyde 3-phosphate into 1, 3-diphosphoglyceric acid is of interest from various points of view. In the whole of EM pathway, this is the only oxidative step, which involves the reduction of the coenzyme  $\text{NAD}^+$  to produce NADH. Secondly, this step involves the coupling of dehydrogenation and phosphorylation. This reaction is catalysed by the enzyme glyceraldehyde 3-phosphate

dehydrogenase, which has tetrameric structure and each of its polypeptide chain possesses a sulfhydryl ( $-\text{SH}$ ) group at the active site. The enzyme binds four  $\text{NAD}^+$  molecules, one with each of the polypeptide chain as shown below:

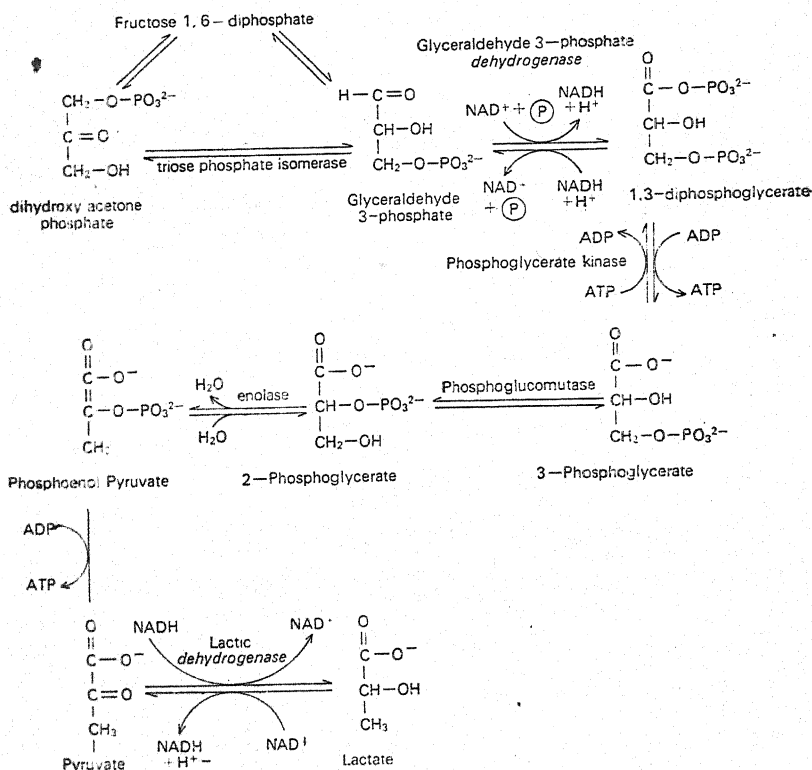
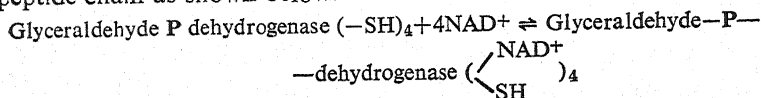


Fig. 7.7. Concluding steps of E.M. pathway.

During its action, the enzyme possibly forms a complex with the substrate, and its sulfhydryl group gets covalently linked to the aldehyde carbon of glyceraldehyde 3-phosphate. This facilitates the oxidation of the aldehyde carbon into an acyl group by the closely lying  $\text{NAD}^+$  which is reduced to  $\text{NADH}$ . The reduced coenzyme  $\text{NADH}$  still remains attached to the enzyme and is soon reoxidized by a free molecule of  $\text{NAD}^+$ .

Alongwith this oxidative phase, a phosphorylative step also operates in this reaction during which an inorganic phosphate is linked to the acyl carbon, producing 1, 3-diphosphoglyceric acid. However, the phosphate ester at carbon 1 possesses unusually high energy of hydrolysis (ca-11,000 cal/mol) and, therefore, in the next step this phosphate group is transferred to a molecule of ADP, producing thereby an ATP and 3-phosphoglyceric acid. This phenomenon, *i.e.* the formation of high-energy phosphate derivative of the substrate followed by its utilization in the synthesis of an ATP, is referred to as substrate-level-phosphorylation. Before a second substrate-level-phosphorylation sets in, the 3-phosphoglyceric acid is converted into 2-phosphoglyceric acid under the influence of phosphoglyceromutase. A dehydration of this compound catalyzed by enolase, causes the formation of a double bond between carbon 2 and 3 of the resulting compound, phosphoenolpyruvic acid. The closely adjacent phosphate ester, therefore, develops a high energy of hydrolysis which drives the transfer of the phosphate group to ADP, thus forming ATP and pyruvic acid, and accomplishes a second substrate-level-phosphorylation.

The complete EM pathway thus involves the degradation of one molecule of glucose into 2 molecules of pyruvic acid. For this the total input (besides one glucose molecule) is four molecules of ADP and two each of phosphoric acid and ATP. The total output consists of two molecules each of NADH, pyruvic acid, ADP and water as well as four molecules of ATP. The net input and out-put is therefore, as under (Fig. 7.8).

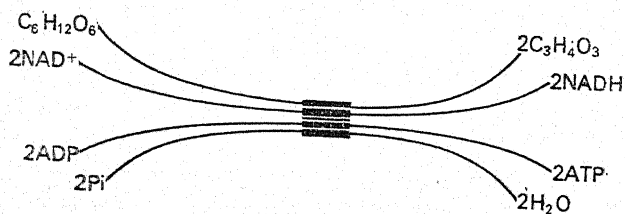
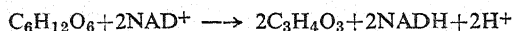


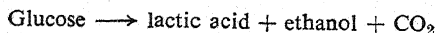
Fig. 7.8.

During this sequence, altogether four phosphorylative steps take place; two of the phosphate groups are supplied by phosphoric acid and the other two by ATP. All these four phosphate groups by substrate level phosphorylation ultimately contribute to the formation of four ATP molecules. But the net gain is only two molecules of ATP

per hexose unit. However, the cell gains 3 ATP molecules, if the initial substrate is glycogen (or starch), because in that case formation of fructose diphosphate requires only a single ATP molecule. The EM pathway also involves the transfer of two hydrogen atoms to the coenzyme  $\text{NAD}^+$ , producing two molecules of reduced coenzyme NADH:



The reduced coenzyme NADH may be reoxidized by different hydrogen acceptors under different conditions. Under aerobic conditions, the ultimate hydrogen acceptor is oxygen, but the electrons are transported through a series of enzymes, called the respiratory chain. During this process enough energy is liberated which is utilized for the synthesis of ATP molecules from ADP and inorganic phosphate. Under aerobic conditions, therefore, the EM pathway furnishes higher number of ATP molecules, as is discussed later. On the other hand, the net gain of energy from the entire EM pathway is restricted to two molecules of ATP only, if the respiration is occurring under anaerobic conditions. Because, in that case, the pyruvic acid acts as the final hydrogen acceptor and oxidizes the NADH, while it is itself reduced to lactic acid. Anaerobic utilization of carbohydrates with formation of lactate in fungi is known chiefly for some Phycomycetes, although presence of a little oxygen is necessary. Two types of metabolic patterns have been reported in lactate producing phycomycetous fungi. The lower Phycomycetes like *Allomyces arbuscula*, *Blastocladia pringsheimii* etc. appear to be homolactic fermentor and are able to convert all the glucose into lactate under anaerobic conditions (Cantino, 1949, 51; Ingraham and Emerson, 1954; Golueke, 1957). *Rhizopus* spp., however, anaerobically produce lactic acid only in 50 per cent proportion alongwith equimolar amounts of ethanol and carbon dioxide. Although Waksman and Foster (1938) have proposed the overall reaction of heterolactic fermentation as follows, yet the mechanism by which the three products are formed in equimolar amounts remains to be understood:



Also, it is not yet known as to why the proportion of lactate production is increased under aerobic conditions. Of course, conversion of pyruvate to lactate has already been demonstrated (Foster, 1949) and radioisotopic studies suggest that a direct reduction of pyruvate occurs (Carson *et al.* 1951 a).

The recently described aquatic Leptomitales *Aqualinderella ferment-*



*tans* (Emerson and Weston, 1967) has been reported by Held (1970) as a homolactic fermentor, producing lactate both under aerobic and anaerobic conditions. The fungus exhibited certain unusual characteristics, and its growth-rate and fermentative activity were not affected by oxygen, rather it required a high level of  $\text{CO}_2$  (Emerson and Held 1969).

Formation of ethyl alcohol during anaerobic utilization of carbohydrates is more common among fungi, especially among the *Mucorales* and *Fusaria*. Wehmer (1907), Czapek (1922), Foster (1949) and Cochrane (1958) have reviewed earlier literature on ethanol production by species of *Aspergillus*, *Fusarium*, *Mucor* and *Penicillium*. Many more fungi, including *Ashbya gossypii* (Mickelson 1950) *Rhizopus* spp. (Harter and Weimer, 1921; Waksman and Foster, 1938; Ward *et al.* 1938) *Diplodia tubericola* (Harter and Weimer 1921), *Dematium pululans* (Sumiki, 1929), *Mortierella* spp. (Perlman, 1950) *Neurospora crassa* (Perlman, 1949), *Stemphylium radicinum* (Lopez-Ramos and Schubert 1955), as well as several wood decaying Basidiomycetes, produce ethyl alcohol in culture solution. *Fusarium* spp. are the most prolific producers of ethanol.

Production of ethanol and  $\text{CO}_2$  has been known for yeasts and the sequence of reaction is generally referred as alcoholic fermentation. It involves the EM pathway upto the formation of pyruvic acid, which is further degraded into a 2-carbon compound, the acetaldehyde through a decarboxylation reaction. This step requires the presence of a pyruvate decarboxylase and the coenzyme thiamine pyrophosphate. Subsequently the acetaldehyde acts as the final hydrogen acceptor and its reduction into ethyl alcohol is achieved by the oxidation of NADH under the catalytic influence of alcohol dehydrogenase. The oxidized coenzyme  $\text{NAD}^+$  is once again available for utilization in the EM pathway.

That formation of ethanol in filamentous fungi follows the same sequence as in yeasts is now almost evident. The product of EM pathway, the pyruvic acid, is formed and may be isolated from many fungi and actinomycetes (Cochrane, 1952; Hida, 1935; Ramachandran and Walker, 1951, 52) Semeniuk, 1944. Its decarboxylation into acetaldehyde is indicated by the latter's presence in the mycelial mats of various fungi (Bolcato and Tono, 1939; Lockwood *et al.* 1938; Nord and Sciarini, 1946; Schmidt Lorenz, 1956) Semeniuk, 1944 as well as decrease in the accumulation of pyruvate by the addition of thiamine (Wirth and Nord, 1942; Strauss, 1952) a component of the coenzyme thiamine pyrophosphate, which helps in the decarboxylation of pyruvic acid. Recent demonstration of the enzyme alcohol-dehydro-

genase in *Rhizopus* (Margulies and Vishniac, 1961) and subsequently in twenty-four other Mucorales (Gleason, 1971; Stuart, Force and Gleason, 1913) has further consolidated that ethanol production in filamentous fungi more or less follows a route similar to that in the yeasts.

Under aerobic conditions, however, oxidation of NADH is accomplished by electron-transport and oxidative phosphorylation, the final hydrogen acceptor being oxygen, and the total aerobic energy gain upto the pyruvic acid stage being eight ATP molecules. Further aerobic degradation of pyruvic acid yields many more ATP molecules, as has been indicated in the next two chapters.

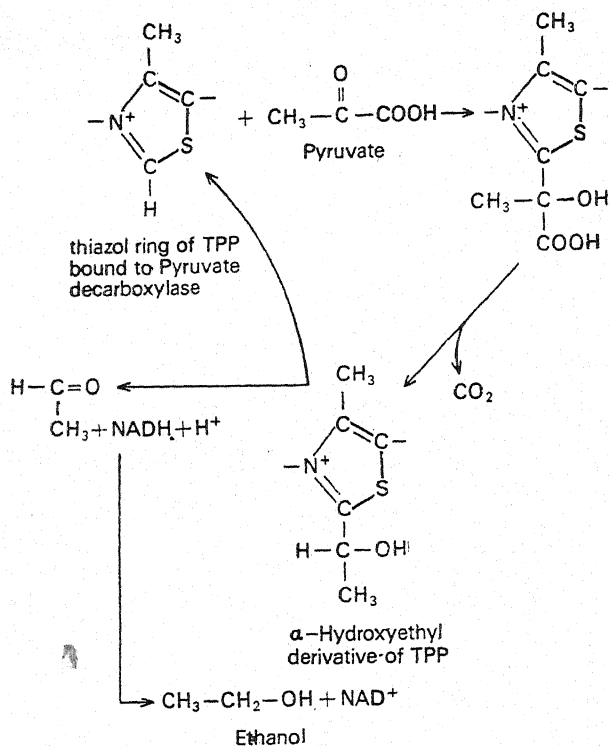


Fig. 7.9. Pyruvate degradation through fermentation.

### Hexose Monophosphate Pathway

An alternative sequence of reactions leading to the break-down of glucose from the monophosphate level was indicated by Warburg and Christian (1932, 1936, 1937) as well as by Dickens (1938). Later

Horecker (1953), Racker (1954, 57) and various other investigators have contributed to the elucidation of this pathway, variously called as hexose-monophosphate oxidative shunt, the direct oxidative pathway or the phosphogluconate oxidative scheme.

Among the various reaction sequences operating through or part of the HMP pathway, the pentose-phosphate pathway is the best known and is quite common among fungi. The other two schemes using HMP pathway have only recently been investigated and evidences of their operation have been recorded in a limited number of fungi. Discussion of the three pathways follows in the sequence of their distribution among fungi.

**Pentose phosphate sequence.** This alternative pathway of glucose catabolism is chiefly aerobic, although some evidences of an analogous fermentative pathway has also been presented by Gunsalus *et al.* (1955). The PP pathway shown in Figs. 7.10, 7.11, 7.12, comprises two distinct phases, as has been indicated by Racker (1957),

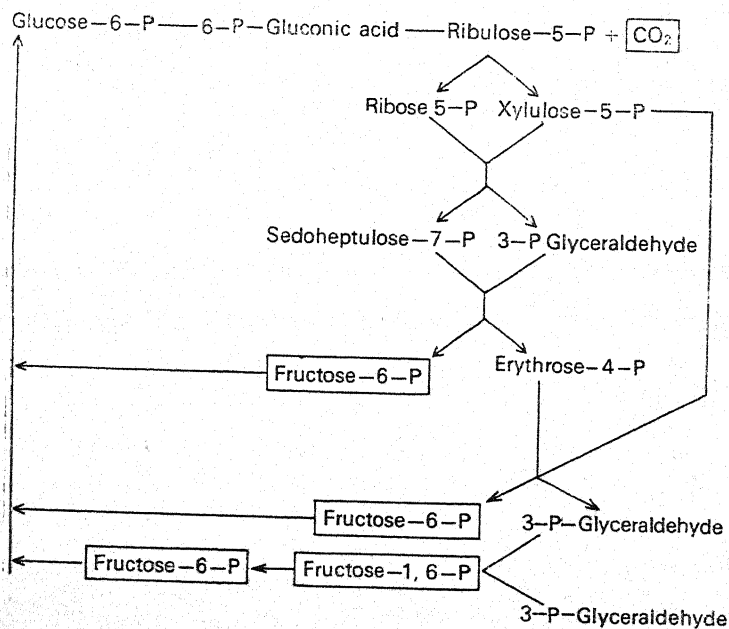


Fig. 7.10. The overall pentose-phosphate cycle.

*viz.* (i) the oxidative phase, in which the hexose molecule undergoes partial combustion, producing pentose sugar and carbon dioxide, and (ii) the non-oxidative phase involving the regeneration of the hexose

sugar, through a number of phosphorylated intermediate sugars with 3-, 4-, 5-, 6- or 7-carbon atoms.

The oxidative phase (Fig. 7.11) is initiated with the oxidation of glucose 6-phosphate to 6-phosphogluconolactone, in presence of the enzyme glucose 6-phosphate dehydrogenase, where the coenzyme  $\text{NADP}^+$  serves as the hydrogen acceptor. In the next step the enzyme lactonase catalyzes the formation of 6-phosphogluconic acid by adding one molecule of water to 6-phosphogluconolactone. A second oxidative step involves an oxidative decarboxylation of the 6-phosphogluconic acid, in which the carbon-1, *i.e.* the aldehyde carbon of original glucose molecule is oxidized to carbon dioxide, with the

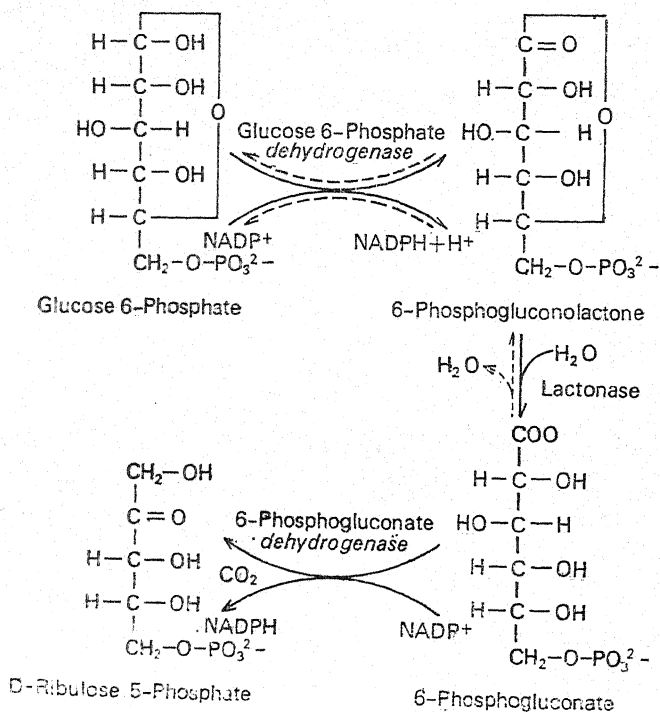
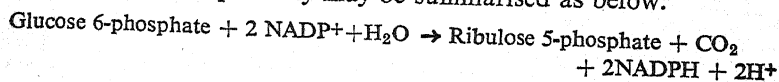
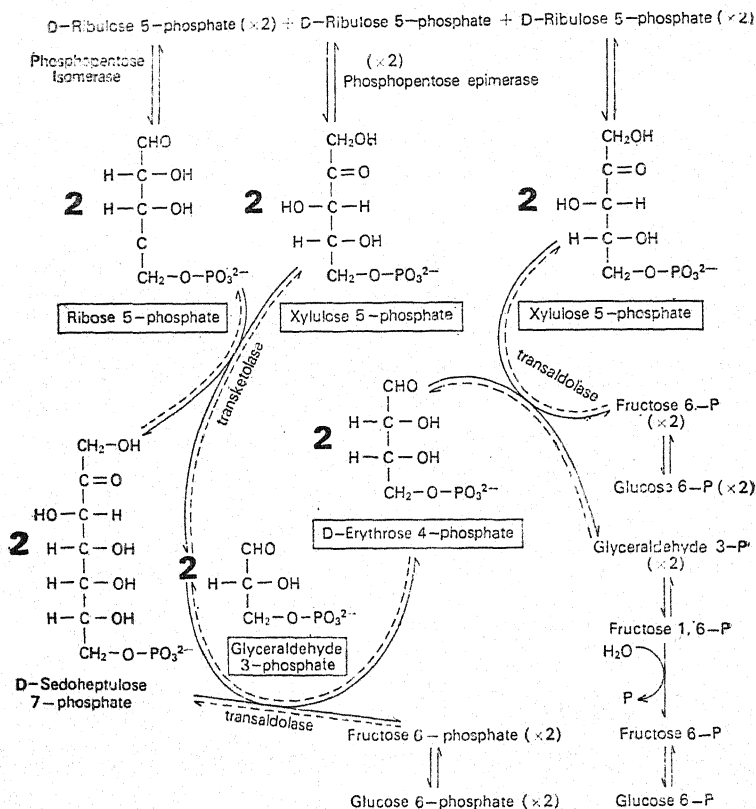


Fig. 7.11. PP pathway.

result that a pentose sugar, ribulose 5-phosphate is formed. This reaction is catalyzed by the enzyme 6-phosphogluconate dehydrogenase and the reduced coenzyme  $\text{NADPH}$  is produced. The oxidative phase of the PP pathway may be summarised as below:



The second phase involves complex pentose-interconversions under specific enzyme action. Such enzymatic transformations of ribulose



5-phosphate into isomeric pentoses are the characteristic features of

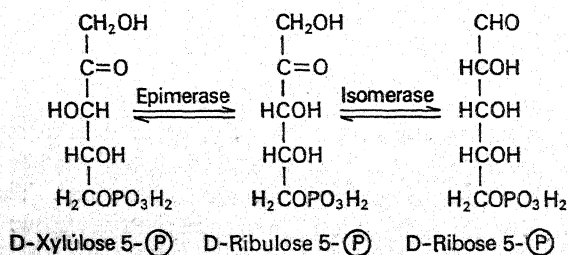


Fig. 7.13. Pentose interconversions.

the PP pathway. Enzymes, like epimerases, isomerases, transketolase and transaldolases play their part in these rearrangements.

The phosphopentose epimerase brings about epimerisation about C-3 and thus causes the interconversion of D-ribulose 5-phosphate and D-xylulose 5-phosphate. Similarly, phosphopentose isomerase catalyzes the interconversion of ketopentose and aldopentose forms. Transketolase enzyme is known to carry out the transfer of a 2-carbon unit, the active glycolaldehyde, from a keto-sugar to an aldose sugar. The D-xylulose 5-phosphate serves here as the source of the 2-carbon unit and the transketolase reaction leads to the formation of D-sedoheptulose 7-phosphate as shown in Fig. 7.14.

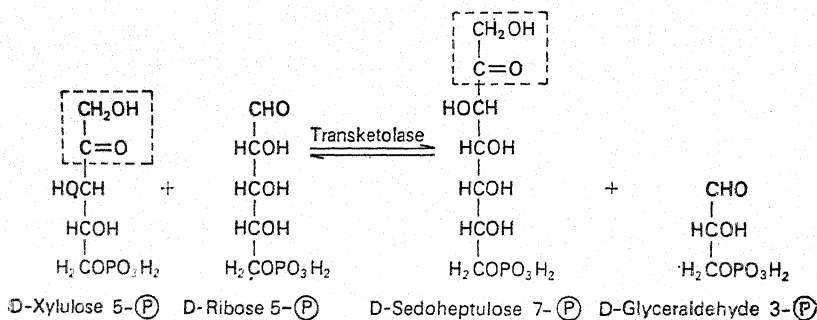


Fig. 7.14.

The coenzyme thiamine pyrophosphate is essential for this reaction, as it is thought to form a complex with the glycolaldehyde moiety and helps in its transfer to the aldose, in the following way:

- (i)  $\text{D-xylulose 5-(P)} + \text{TPP} \rightarrow [\text{D-xylulose 5-(P)-TPP}]$   
 $\rightarrow \text{Dihydroxyethyl-TPP} + \text{D-glyceraldehyde-3-(P)}$   
 (ii)  $\text{D-Ribose 5-(P)} + \text{Dihydroxyethyl-TPP} \rightarrow \text{D-Sedoheptulose 7-(P)} + \text{TPP}$

Similarly, the enzyme transaldolase is active in the transfer of carbon atoms 1, 2 and 3 of a ketose phosphate to carbon-1 of an aldose, in the following manner (Fig. 7.15).

The erythrose 4-phosphate formed this way may undergo a transketolase reaction and add a 2-carbon unit to produce a fructose 6-phosphate (Fig. 7.16).

All these complex rearrangement ultimately lead to the regeneration of glucose 6-phosphate. Such conversion of pentose phosphate to hexose phosphate and formation of intermediates like sedoheptulose phosphate etc. are easily demonstrated in cell free preparations of fungi as well as actinomycetes (Cochrane and Hawley, 1956; Newburgh, Cloridge and Cheldelin, 1955; Sih *et al.* 1957).

The enzymes referred to above are unique to this pathway. However, certain enzymes of the EM pathway are also functional in this scheme. These include, (i) triosephosphate isomerase catalysing interconversion of phosphoglyceraldehyde and dihydroxyacetone phosphate, (ii) aldolase, which controls the synthesis of fructose 1, 6-diphosphate from the triose phosphates (iii) diphospho fructose phosphatase to hydrolyze fructose 1, 6-diphosphate to fructose 6-phosphate, and (iv) hexose phosphate isomerase for the conversion of fructose 6-phosphate to glucose 6-phosphate.

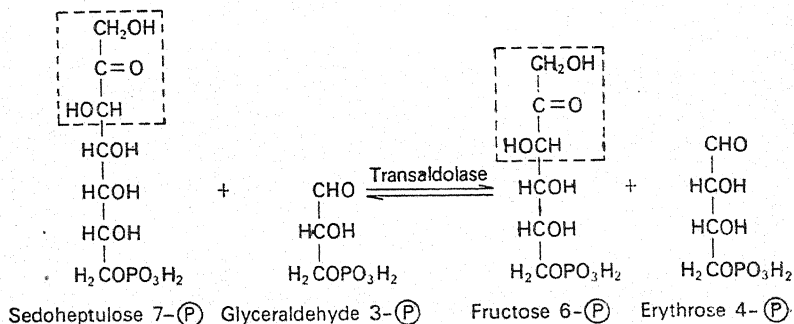


Fig. 7.15.

From all these reactions the cyclic nature of this pathway may easily be appreciated, in which the hexose enters continuously, while

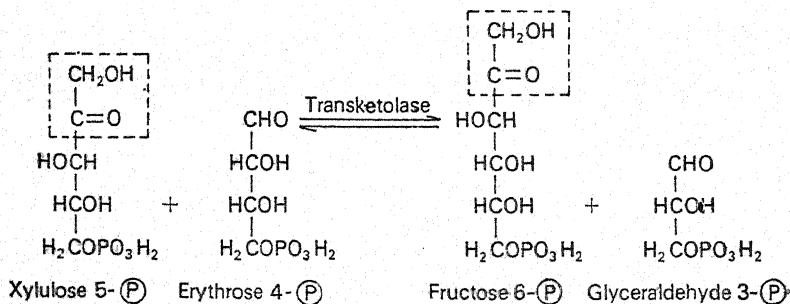
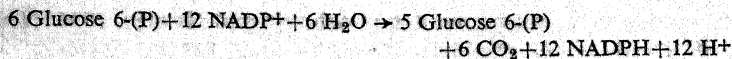
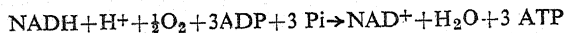


Fig. 7.16.

CO<sub>2</sub> emerges as the sole carbon compound. Thus, in each operation of the cycle although six hexose molecules are utilized, five of them are regenerated along with 6-carbon dioxide molecules as the degradation product. The overall input and output of this sequence may be written as under:



The output includes the all important 12 molecules of the reduced coenzyme NADPH, produced during the oxidative phase of the pathway. Since 5 molecules of glucose 6-phosphate are regenerated, therefore all the 12 NADPH may be considered as the product of complete oxidation of the equivalent of only one glucose molecule. These 12 NADPH molecules may be taken as equivalent of 36 molecules of ATP on the basis of the known reaction of its counterpart NADH:



However, this type of reaction for the oxidation of NADPH is not known. The various possibilities regarding its reoxidation include, (i) oxidation by respiratory chain via NAD, (ii) oxidation at the expense of oxygen and (iii) oxidation through its reducing action in various cellular anabolic processes.

The first possibility is based on evidences obtained from mitochondrial coenzymes. Mitochondria are known to contain both NAD and NADP coenzymes, and in many cases an enzyme transhydrogenase which catalyzes the transfer of hydrogen atoms to and from NAD and NADP. This has led to the assumption that mitochondrial NADPH is reoxidised by the respiratory chain through NAD (Fig. 7.17). Whether the same is true for the NADPH produced in the PP pathway operating outside mitochondria, is yet to be ascertained.

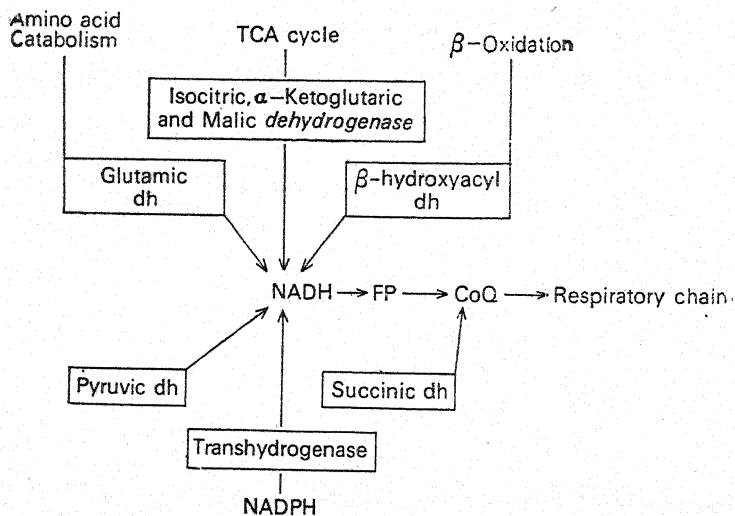
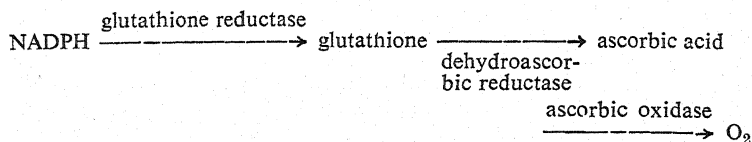


Fig. 7.17. Reduction of NAD<sup>+</sup> in mitochondria.



On the contrary, soluble systems are known which may lead to the oxidation of NADPH at the expense of oxygen, although none has been demonstrated to operate *in vivo*. One of the best known system proceeds as follows:



However, it seems more likely that much of the NADPH produced during the PP pathway are utilized in reductive biosynthetic reactions.

The PP pathway has a number of interesting features, which need mention. This pathway affords a method for the total combustion of glucose without the participation of citric acid cycle. It provides for the synthesis of NADPH, which has essential role, in fatty acid synthesis and various hydroxylases. The NADPH, in fact may be regarded as 'reducing power' in such a form that cannot be siphoned off through electron transport chain, and therefore may be utilized for synthetic and other purposes. Besides, this pathway serves a major role in the synthesis of pentoses, tetrose and 7-carbon sugar. The formation of ribose is particularly significant, in view of its role in the synthesis of nucleic acid. Also, the pathway does not require any further ATP after the formation of glucose 6-phosphate.

*Entner Doudoroff sequence.* This is another sequence of reactions, belonging to the hexose monophosphate pathway and first reported in *Pseudomonas saccharophila* by Entner and Doudoroff (1952). The ED pathway has some common steps with the PP pathway and the two sequences diversify only after the formation of 6-phosphogluconate. Further steps of the ED pathway include (i) dehydration of the 6-phosphogluconate leading to the formation of 2-keto-3-deoxy-6-phosphogluconate, and (ii) cleavage of this product into glyceraldehyde 3-phosphate and pyruvate. Obviously, these two steps are catalyzed by enzymes which are specific to this pathway. These are (i) 6-phosphogluconate dehydratase and (ii) 2-keto-3-deoxy-6-phosphogluconate aldolase. The reactions of this sequence are schematized in Fig. 7.18.

Glyceraldehyde 2-phosphate may be converted into yet another molecule of pyruvate through the known steps of EM pathway and may yield two molecules of ATP. Thus the net gain from degradation of each molecule of glucose by way of ED pathway, comes to a mere one molecule of ATP. The single molecule of reduced co-

enzyme NADPH may yield an equivalent of another 3-molecules of ATP. Thus an equivalent of 4 ATP molecules is gained by way of degradation of each molecule of glucose upto the level of pyruvate, through this pathway.

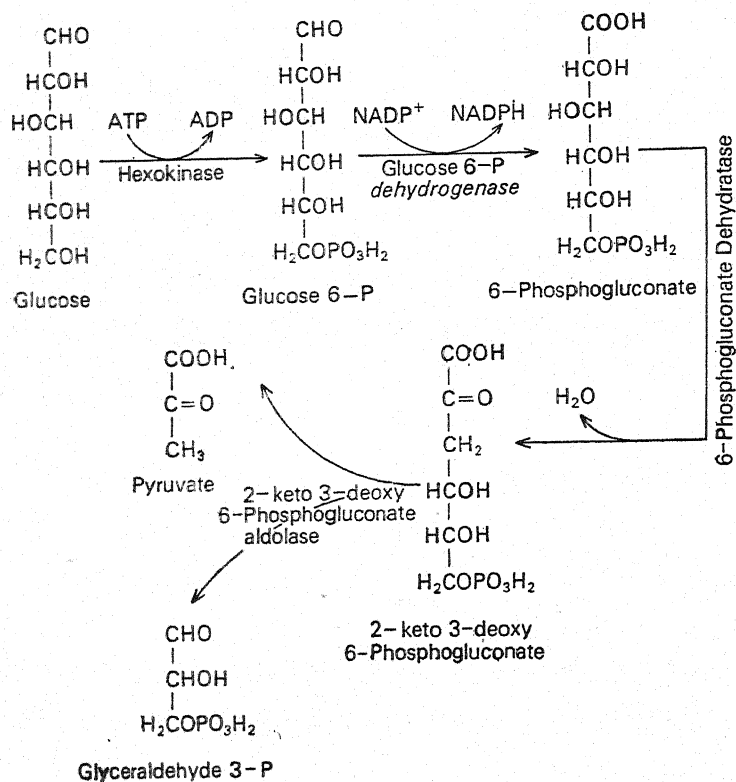


Fig. 7.18. Enter-Doudoroff sequence.

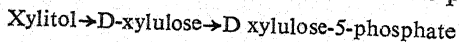
**Glucuronate-Xylulose cycle.** Yet another sequence of glycolytic reactions following a part of the HMP pathway is the glucuronate xylulose cycle or uronic acid pathway reported, mostly in animals. The pathway generally proceeds along the steps shown in Fig. 7.19 and although its quantitative significance is not yet ascertained, yet it seems to be of minor nature in fungi.

In this pathway, glucose 6-phosphate is first converted into glucose 1-phosphate under the influence of phosphoglucomutase. This is followed by a reaction catalyzed by the enzyme **UDPG pyrophosphorylase**, during which glucose-1-phosphate and uridine triphosphate



tic action of a NAD-dependent UDPG dehydrogenase. The next reaction involves the inversion of the carbon-chain which gives rise to L-gulonic acid. During this step the glucuronic acid is reduced with the help of NADPH to L-gulonic acid, which is a direct precursor of ascorbic acid. This seems to be one of the major significance of this cycle, and enhanced synthesis of ascorbic acid has often been ascribed to the operation of this cycle. *Aspergillus niger* when grown on gulonolactone incorporated media, yielded three times higher quantity of ascorbic acid from its mycelium, besides an 18% increase in the mycelial dry-weight. However, this is too indirect an evidence to suggest an operative uronic acid pathway among fungi (Sastry and Sarma, 1957).

The gulonic acid is further oxidized with the help of the coenzyme  $\text{NAD}^+$  to produce 3-keto-L-gulonic acid. The next step involves the decarboxylation of the acid resulting in the removal of one molecule of  $\text{CO}_2$  and formation of L-xylulose. Thus, this cycle also involves the removal of one  $\text{CO}_2$  molecule, like the PP pathway but the carbon lost in this cycle is C-6 instead of C-1. The L-xylulose has to be converted into its D-isomer before it may be utilized via pentose phosphate pathway. Obviously, the next few steps of the uronic acid cycle accomplishes this feat through the formation of xylitol. Conversion of L-xylulose into xylitol requires the participation of NADPH, which acts as the reducing agent. In the very next step reoxidation of xylitol into D-xylulose takes place, the coenzyme involved this time being  $\text{NAD}^+$ . Phosphorylation of D-xylulose by the ATP leads to the formation of D-xylulose 5-phosphate, which then enters the PP pathway. In slime molds, experiments with labeled glucose indicated that during two developmental stages, the yield of  $^{14}\text{CO}_2$  was more from the glucose 6- $^{14}\text{C}$  (Wright, 1963). Although with our present knowledge such results could only be attributed to an operative uronic acid cycle, but presence of certain key enzymes of this pathway could not be established in slime molds and, therefore, this problem cannot be considered as fully resolved. However, some of the characteristic enzymes of this pathway have been found in *Penicillium chrysogenum* and some other filamentous fungi (Chiang and Knight, 1960) as well as in yeasts (Horecker, 1962 a). These fungi were found to operate an interesting reductive pathway of pentose metabolism, atleast a part of which follows the sequence of uronic acid cycle. The steps common to both the pathways are:



Through these steps an L-pentose like L-arabinose may be converted

into D-xylulose 5-phosphate through xylitol.

Occurrence of the preferential oxidation of the 6th carbon atom of yet another hexose has also been reported. Avigad *et al.* (1961) reported that *Polyporus circinatus* produces a D-galactose oxidase enzyme which oxidizes the 6th carbon of D-galactose and produces, D-galactodialdose. Information on its further metabolism is, however, lacking;

Despite all these indications, further investigations are necessary before the occurrence of this pathway among fungi is firmly established.

### Occurrence and Role of Pathways in Fungi

Studies relating to carbohydrate catabolism in fungi have achieved significant break-through during the last two decades mainly due to the use of radioisotopic technique with intact cells, and studies with isolated enzymes. Methods employed by different workers to establish the existence and significance of different pathways have found adequate treatment in the accounts given by Foster (1949), Cochrane (1958), Lamanna and Mallette (1959) as well as Blumenthal (1965). Generally the following seven techniques have found use in studies dealing with fungal respiration: (i) Measurement of carbon and oxidation-reduction balance; (ii) estimation of growth on different carbon and energy sources; (iii) manometric studies; (iv) inhibitor-studies; (v) use of biochemical mutants; (vi) enzymatic studies; and (vii) radioisotopic investigations. Of these the last two methods, involving the occurrence of specific enzymes and intermediates in cell extracts and studies on distribution of isotopic carbon in CO<sub>2</sub> or some intermediates have proved more helpful as far as the respiratory pathways and their occurrence are concerned. Other techniques have either proved inadequate for such studies (*e.g.* oxidation-reduction measurement), or have yielded indirect evidences due to various limitations (*e.g.* endogenous respiration) or have found only limited use, for the investigation of glycolytic mechanisms (*e.g.* use of biochemical mutants).

Present knowledge of the respiratory metabolism of fungi indicates that the following four glycolytic pathways are operative in fungi namely (i) the Embden-Meyerhof (EM) pathway, (ii) Pentosephosphate (PP) pathway, (iii) Entner-Doudoroff (ED) pathway, and (iv) Glucuronate-xylulose pathway. Of these, EM pathway is apparently the most widespread while glucuronate-xylulose pathway is yet to be conclusively demonstrated in fungi. Of the remaining two, PP pathway is more frequent than ED pathway.

The ten enzymes associated with the EM pathway (Table 7.1)

occur widely in fungal mycelia and spores which indicates that the fungal cells have the potential to use this pathway. The only enzyme specific to this pathway is phosphofructokinase and its presence in a cell may be a definite indication of EM pathway. However, its apparent absence have often been improperly interpreted. As suggested by Blumenthal (1965) negative evidences, such as inability to isolate or detect an enzyme or some intermediate products should not alone form the basis of any drastic conclusion. This is because various factors may interfere with their demonstration, namely labile nature of the enzyme as was the case with *Penicillium chrysogenum* Sih and Knight, 1956; Sih *et al.* 1957) as well as *Microsporium canis* (Chattaway *et al.* 1960); presence of some inhibitor, as reported by Khanna and Tiwari (1963) in *Aspergillus niger* spore extract, which inhibited hexokinase and phosphoglucomutase, etc. Similar negative evidences like, apparent lack of phosphoglyceric acid as well as absence of the enzyme phosphoglycerate mutase in *Fusarium* (cf. Foster, 1949; Cochrane, 1956, 58) had led Nord and Weiss (1951) to erroneous conclusions, when they postulated that *Fusarium* lacked the EM pathway of glucose catabolism. However, Cochrane (1956) and Heath *et al.* (1956) were soon able to demonstrate the occurrence of EM pathway in intact cells of *Fusarium*, which not only settled this particular question but also falsified a concept formulated by Nord and his associates that in the filamentous fungi phosphoryla-

TABLE 7.1

ENZYMES INVOLVED IN DIFFERENT PATHWAYS OF  
GLUCOSE-CATABOLISM

Enzymes	Pathways		
	EM	PP	ED
1. Hexokinase	+	+	+
2. Phosphohexose isomerase	+	+	—
3. Phosphofructokinase	+	—	—
4. Aldolase	+	+	—
5. Triosephosphate isomerase	+	+	+
6. Phosphogluceraldehydehydrogenase	+	+	+
7. Phosphoglucerate kinase	+	+	+
8. Phosphoglucerate mutase	+	+	+
9. Enolase	+	+	+
10. Pyruvate kinase	+	+	+
11. Glucose-6-P-dehydrogenase	—	+	+
12. 7-phosphogluconolactonase	=	+	+
13. Phosphogluconate dehydrogenase	—	+	—
14. Ribose-P isomerase	—	+	—

15. Ribulose-P epimerase	—	+	—
16. Transketolase, glyceraldehyde transferase	—	+	—
17. Transaldolase, dihydroxy acetone transferase	—	+	—
18. 6-phosphogluconate dehydratase (or dehydrase)	—	—	+
19. 2-keto-3-deoxy-6-phosphogluconate aldolase	—	—	+

tion had a little part to play, and some non-phosphorylative pathway was the primary mechanism for glucose catabolism.

However, a few exceptional conditions have been reported among fungi, where the EM pathway seems to be completely inoperative. These are *Caldariomyces fumago* (Ramachandran and Gottlieb, 1963) and spores of *Tilletia caries* (Newburgh and Cheldelin, 1958). Mycelium of *T. caries*, however, degrades 66% of its hexose phosphate through EM pathway (Newburgh and Cheldelin, 1958). It is interesting to note that *T. caries* so far appears to be the only fungus which is known to operate EM, PP and ED pathways, the first two in the mycelium, while the last one in spores.

The pentose phosphate pathway comes only next to the EM pathway and is the best known alternative sequence of glucose phosphate degradation among fungi and accounts for nearly 40% glucose dissimilation. Of the sixteen enzymes associated with this pathway (Table 7.1) as many as five are specific to this sequence namely phosphogluconate dehydrogenase, ribose-P isomerase, ribulose-P epimerase, transketolase, and transaldolase. Similarly, NADP is the coenzyme, which is usually functional in the oxidative phase of the PP pathway. Enzymatic studies indicate that these enzymes may be widely distributed among fungi. Transketolase and transaldolase have so far been demonstrated in *Tilletia caries* (Newburgh *et al.* 1955), *Penicillium chrysogenum* (Sih *et al.* 1957), *Aspergillus niger* (McDonough and Martin, 1958), *Claviceps purpurea* (McDonald *et al.* 1960) and *Ustilago maydis* (Gottlieb and Caltrider, 1963). In fact these two enzymes have been detected in all the cells studied so far and it has often been considered to be of universal distribution (Horecker, 1962). Similarly, glucose 6-P and 6-phosphogluconate dehydrogenases were found to be present in all the nine fungi surveyed by Dowler *et al.* (1963). As regards NADP, Hochster (1957) observed that in *Aspergillus flavus-oryzae* NADP-linked glucose 6-P and 6-phosphogluconate dehydrogenases were present, together with their NAD-linked counterpart. The former was found to be present and detectable from the very beginning of growth of the fungus, while the latter could not be detected before the third day. However, Eagon (1963) suggests that this coenzyme may act as the limiting factor in the operation of the PP pathway in fungi and other microorganisms.



From our present knowledge the Entner-Doudoroff (ED) pathway appears to be of very limited distribution, and is known to operate in a few bacteria and only two fungal species namely *Caldariomyces fumago* and *Tilletia caries* spores. In spores of *T. caries* ED pathway is solely responsible for glucose catabolism, whereas in *C. fumago* it accounts for 65% of glucose degradation, although this fungus lacks the enzyme hexokinase, and appears to use a bit modified ED sequence (Blumenthal, 1965). Eleven enzymes are involved in the ED pathway of which two, viz., 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase are specific to this pathway, and their presence is highly suggestive of the ED sequence. However, presence of these enzymes are yet to be established even in *T. caries* and *C. fumago*, which are the only fungi where other evidences indicate an operative ED pathway. A few reports instead indicated their absence in *Aspergillus niger* (McDonough and Martin, 1958; Geser, 1962). One of the enzymes namely 6-phosphogluconate aldolase has been listed to be formed by *Aspergillus flavus-oryzae* (Burnett, 1968). Obviously, further studies are required for a more accurate estimation of this pathway among fungi.

Operation of yet another hexose-pentose cycle, namely glucuronate-xylulose pathway (also called glucuronic acid cycle) is not yet firmly

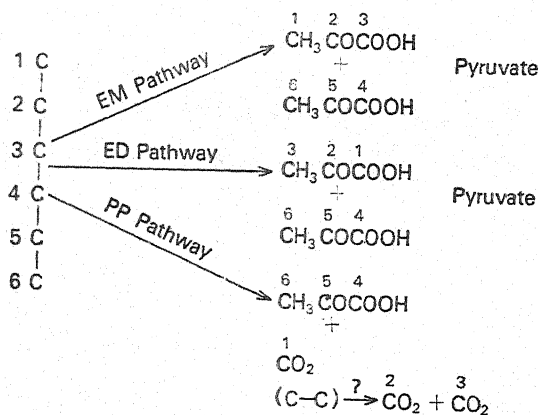


Fig. 7.20. Distribution of carbon atoms in different pathways.

established in fungi. As already indicated some limited evidences have been adduced in a few filamentous fungi viz. *Aspergillus niger* (Sastry and Sarma, 1957), *Penicillium chrysogenum* and others (Chiang and Knight, 1960) but they are too indirect to reach any definite conclusion and, therefore, much more sustained efforts are required in this direction.



Although, many of the enzymatic studies referred to above were helpful in investigations pertaining to different pathways and their occurrence among fungi, they did not clearly establish their operation. The radioisotopic technique, on the other hand, proved more useful for conclusive evidences. This is because, EM, PP and ED pathways exhibit marked differences in the distribution of different carbon atoms in the degradation products, as is shown in Fig. 7.20.

Although more than one catabolic pathways of glucose utilization have been found to be operative in the majority of fungi studied so far (Table 7.2) but the common occurrence of a number of enzymes

TABLE 7.2  
OCCURRENCE OF GLYCOLYTIC PATHWAYS IN FUNGI

Fungi	% Occurrence of Pathways			References
	EM	PP	ED	
<i>Candida utilis</i>	50-96	4-50	—	Blumenthal <i>et al.</i> (1964)
<i>Saccharomyces cerevisiae</i>	83-100	0-27	—	Do
" "	88	12	—	Wang <i>et al.</i> (1956, 1958 a)
" "	94-96	4-6	—	Chen (1959 a)
<i>Rhizopus oryzae</i>	100	—	—	Gibbs and Gastel (1953)
<i>Rhizopus</i> MX	100	—	—	Margulies and Vishniac (1961)
<i>Neurospora crassa</i> mycelium	88-99	1-12	—	Blumenthal (1962)
" " conidia	90	10	—	Do
<i>Claviceps purpurea</i>	90-96	4-10	—	McDonald <i>et al.</i> (1960)
<i>Aspergillus niger</i>	78	—	—	Shu <i>et al.</i> (1954)
<i>Penicillium chrysogenum</i>	56-70	30-46	—	Lewis <i>et al.</i> (1954)
" "	42	58	—	Heath and Koffler (1956)
" "	77	23	—	Wang <i>et al.</i> (1958 a),
<i>Penicillium digitatum</i>	77-83	17-23	—	Do Reed and Wang (1959)
<i>Caladariomyces fumago</i>	—	35	—	Ramachandran and Gottlieb (1963)
<i>Fusarium lini</i>	83	17	—	Heath <i>et al.</i> (1956)
<i>Verticillium alboatrum</i>	48	52	—	Brandt and Wang (1960)
<i>Tilletia caries</i> mycelium	66	34	—	Newburgh and Cheldelin
<i>Tilletia caries</i> spores	—	—	100	Do
<i>Tilletia contraversa</i> spores	33	67	—	Do (1959)

in these pathways, provide an interlocking device, which controls the sequence of reactions under specific conditions. Of the ten EM enzymes listed in Table 7.1, seven and nine are also common to PP and ED pathways respectively. Therefore there is a competition for substrate, on whose level depends the direction in which the enzyme

catalyzed reaction proceeds. For example, the enzymes glucose phosphate isomerase, triose-phosphate isomerase and aldolase catalyze the degradation of glucose phosphate into pyruvate, while they play a reversible role in the pentose phosphate pathway. Besides, one pathway may exert inhibitory effect upon the other through its intermediate products. For examples, sedoheptulose-7-phosphate and phosphogluconate of PP pathway have been shown (not in fungi) to inhibit the activity of glucose-phosphate isomerase.

Despite all these, EM pathway has been found to be the primary one in majority of fungi studied so far, followed by PP and ED pathways respectively. In those cases, where PP pathway played a primary role, the EM pathway acted as a secondary one, and where ED pathway was the major route, the PP pathway was the minor one but in no case EM and ED pathways operated together. This apparent incompatibility between EM and ED pathways is however, not yet clearly understood.

From the foregoing discussion it is evident that our present knowledge of different pathways, as well as their quantity of operation in fungi is far from complete. Besides, the few fungi which have been partially investigated belong to the common moulds, and, therefore, much more remains to be worked out as far as glucose catabolism among fungi in general is concerned.

The overall significance of these glycolytic pathways is to furnish the cell with (a) energy, (b) precursors for biosynthetic reactions, and (c) a system of oxidation-reduction reactions through which these precursors are incorporated or converted into synthetic products.

As already indicated, energy supplied by these catabolic pathways are in two metabolically usable forms namely, the energy rich compound ATP (free energy of hydrolysis of each of the two terminal phosphate group ranging from 8500 to—14,500 cal/mol) and the reduced coenzymes NADH and NADPH which on oxidation act as high energy donors, yielding approximately 52,700 cal/mol. The EM pathway and TCA cycle combination produces the maximum number, *i.e.*, 38 ATP molecules through terminal electron transfer and oxidative phosphorylation. Next is the PP pathway which may yield an equivalent of 35 ATP molecules per molecule of glucose. The ED pathway, with a net yield of 4 ATP molecules per glucose molecule degraded upto the level of pyruvate may be treated as the least efficient of these pathways as far as energy liberation is concerned. In respect of the coenzyme, there is a clear distinction between the EM pathway on the one hand and the two HMP pathways on the

other. In the EM pathway the participating coenzyme is  $\text{NAD}^+$  while in PP and ED pathways  $\text{NADP}^+$  is involved. That reduced forms of these two coenzymes serve different functions is now well recognised Blumenthal (1965) has dwelt upon in detail the role of  $\text{NAD}^+$  and  $\text{NADP}^+$  in fungal metabolism. While  $\text{NADH}$  is universally involved in the energy transfer and synthesis of ATP molecules through terminal oxidation and oxidative phosphorylation,  $\text{NADPH}$  seems to be chiefly functional in reductive biosynthetic reactions. May be on this account, that in the cell the  $\text{NAD}^+$  is found largely in the oxidized form, while  $\text{NADP}^+$  occurs entirely as  $\text{NADPH}$  (Horecker, 1962 B). Functional differences of  $\text{NADH}$  and  $\text{NADPH}$  have been emphasized by quite a number of investigators chiefly by their studies on glutamate dehydrogenases of *Neurospora crassa* and a few other fungi. The glutamate dehydrogenase enzyme catalyzes the key reaction of reductive amination of  $\alpha$ -ketoglutarate into glutamate, which incidentally is the link between carbohydrate metabolism and amino acid synthesis. Sanwal and Lata (1961) reported that *Neurospora crassa* contains two different glutamate dehydrogenases, one specific to  $\text{NAD}^+$  while the other to the  $\text{NADP}^+$ . Two different physiological roles have been ascribed to the  $\text{NAD}^+$  and  $\text{NADP}^+$  specific glutamate dehydrogenases (GDH), the  $\text{NAD}$ -GDH performing catabolic, while the  $\text{NADP}$ -GDH the anabolic functions. This is supported by the observation that  $\text{NAD}$ -GDH formation is induced in glutamate containing medium (Sanwal and Lata, 1962) and that allosteric effects are either very feeble or completely lacking in glutamate dehydrogenases of *Neurospora* (Goldin and Frieden, 1971). In the last few years, the fungal glutamate dehydrogenases have received much attention, and either  $\text{NAD}$ -specific GDH or both  $\text{NAD}$ -GDH and  $\text{NADP}$ -GDH enzymes have been detected in different fungi (Dennen and Niederpruen, 1967; Kato *et al.* 1962; Hierholzer and Holzer, 1963; Stevenson and John, 1971).

The pentose phosphate pathway has more definitive role to play as far as supply of precursors is concerned. The PP pathway provides for the source of different pentoses to the cell, including the ribose, which is required for nucleotide and nucleic acid synthesis. Besides, other sugars like sedoheptulose and erythrose are also important products of the PP pathway, particularly the latter one, which is required in the synthesis of aromatic amino acids.

Some other intermediates, like triose-phosphates, hexose phosphates etc. are the common products of all the three glycolytic pathways.

## RESPIRATION—II

Degradation of glucose to pyruvate or lactate through glycolysis has more than one limitation as far as its functioning as an open biological process is concerned. The main short-comings of this process are due to (i) the possible toxic effects of pyruvic acid accumulation, (ii) the limiting action of the coenzyme  $\text{NAD}^+$  due to its continued reduction to  $\text{NADH}$ , and (iii) inadequate free energy gradient, with pyruvic acid still retaining more than four-fifth of the available energy of the carbohydrate source. These shortfalls may be biologically overcome in various ways, as for instance the pyruvic acid, may itself act as an oxidant to reoxidize the  $\text{NADH}$ , and thus may get rid of its own toxicity; or it may get decarboxylated into acetaldehyde, which then may act as the hydrogen-acceptor to reoxidize the  $\text{NADH}$ , etc. However, the best recourse for the improvement of glycolysis as a respiratory process, both chemically and thermodynamically, is the full aerobic oxidation of pyruvic acid. It is perhaps on this account that great majority or most of the aerobes and facultative anaerobes utilize their glycolytic product pyruvate through an entirely aerobic sequence of reactions variously called as the Krebs cycle, tricarboxylic acid cycle or citric acid cycle, atleast under adequate aeration. The primary step in the aerobic oxidation of pyruvate is, however, an oxidative decarboxylation of pyruvic acid leading to the formation of acetyl coenzyme A, through a series of closely knit reactions.

## OXIDATIVE DECARBOXYLATION OF PYRUVIC ACID

The over-all sequence of reaction is not well understood, although appreciable amount of informations have accumulated regarding the co-factors which participate in this sequence. There are atleast five co-factors including thiamine pyrophosphate (TPP) and  $\text{Mg}^{2+}$ , which are also required in the fermentative decarboxylation of pyruvic acid.

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In fact, the initial steps upto the formation of  $\alpha$ -hydroxyethyl derivative of TPP are similar in both fermentative and oxidative decarboxylation of pyruvic acid. The other three co-factors are  $\alpha$ -lipoic

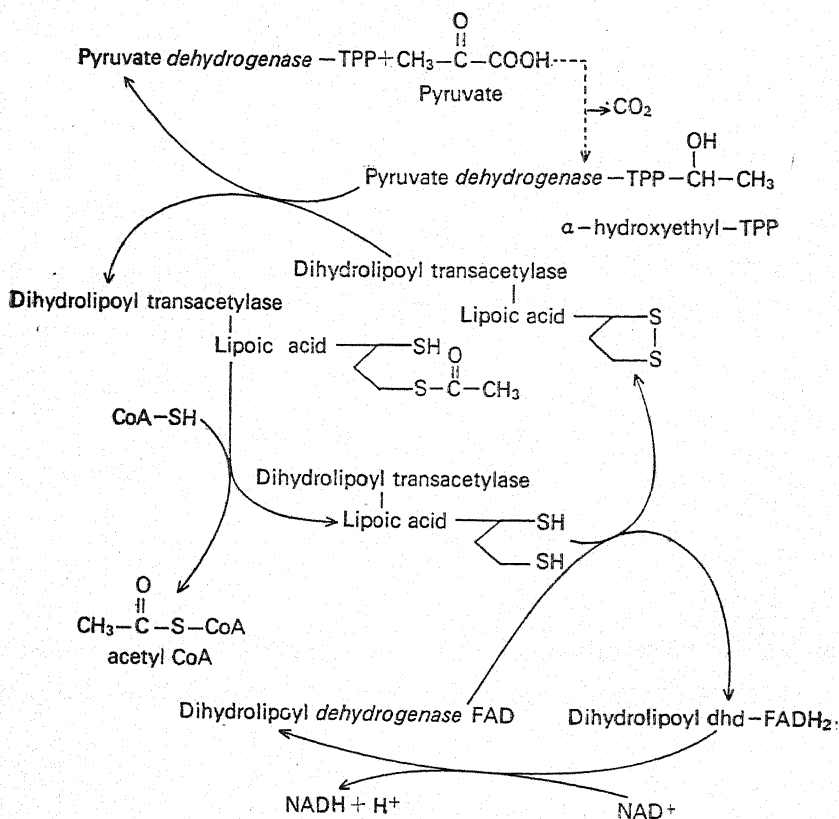
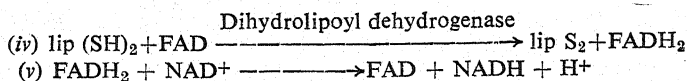


Fig. 8.1. Oxidative decarboxylation of pyruvate.

acid (Lip  $\text{S}_2$ ), coenzyme A (CoA or CoASH) and  $\text{NAD}^+$ . Besides these co-factors, a pyruvate dehydrogenase multienzyme complex comprising three different enzymes catalyzes this sequence. The process is accomplished in the following five different steps:

- (i)  $\text{Pyruvic acid} + \text{TPP} \xrightarrow[\text{Mg}^{2+}]{\text{Pyruvate dehydrogenase}} \text{hydroxyethyl-TPP} + \text{CO}_2$
- (ii)  $\text{hydroxyethyl-TPP} + \text{lip-S}_2 \xrightarrow{\text{Dihydrolipoic transacetylase}} \text{acetyls lip SH} + \text{TPP}$
- (iii)  $\text{acetyl S lip SH} + \text{CoASH} \xrightarrow{\text{acetylase}} \text{acetyl SCoA} + \text{lip (SH)}_2$



The first three steps lead to the decarboxylation of pyruvic acid and the last two are meant to reoxidize the co-factor lipoic acid to its original disulphide form, by the flavoprotein enzyme dihydrolipoyl dehydrogenase, with concomitant reduction of  $\text{NAD}^+$  to  $\text{NADH}$ . The enzyme pyruvate dehydrogenase, which has the TPP bound to it catalyzes the first step of this sequence resulting in the formation of  $\alpha$ -hydroxyethyl-TPP and  $\text{CO}_2$ . In the next step, the two carbon unit ( $\alpha$ -hydroxyethyl) is taken-off from TPP by the enzyme dihydrolipoyl transacetylase-lipoic acid complex and in the process, the hydroxyethyl group is oxidized into an acetyl unit, while the disulphide bond of lipoic acid is reduced to an -SH group. In the subsequent step acetyl unit is transferred to the coenzyme.

### TRICARBOXYLIC ACID CYCLE

Earlier work on further degradation of pyruvic acid was carried out on highly aerobic animal tissues and the first break-through was reported by Szent-Gyorgyi, who established the catalytic effects of some 4-carbon dicarboxylic acids, like succinic, fumaric, malic and oxaloacetic acids on oxidation by breast muscles of pigeon. An equally significant contribution came subsequently from Krebs and Johnson (1937) who reported a similar catalytic role for citric and  $\alpha$ -Ketoglutaric acid. They also showed that oxaloacetic acid could be transformed to citric acid by pigeon breast muscle, and suggested that the two-carbon unit required in such conversion could be furnished by pyruvic acid, which is decarboxylated in the process of condensation. Further, on the basis of inhibitor-studies they developed a cyclic concept of these reactions (the citric acid cycle) which was shown to operate in various kinds of animal tissues, and considered to be absent from yeasts and bacteria (Krebs and Johnson, 1937; Krebs 1943). Actually until a few years back, occurrence of a functional TCA cycle in fungi was very much in doubt, despite some hectic efforts during the last three decades to establish a wide biological distribution of this cycle. Some excellent reviews have since then appeared (Cochrane, 1958; Niederpruem *et al.* 1965; Burnett, 1968) and the presence of a functional TCA cycle in fungi is now well established. As far as the steps of the cycle are concerned, they have been elucidated to an appreciable extent during the last thirty years of research, but a detailed knowledge of the full enzymology of the

cycle is yet to be obtained. The various steps of the cycle (Fig. 8.2) according to the present concept are described below.

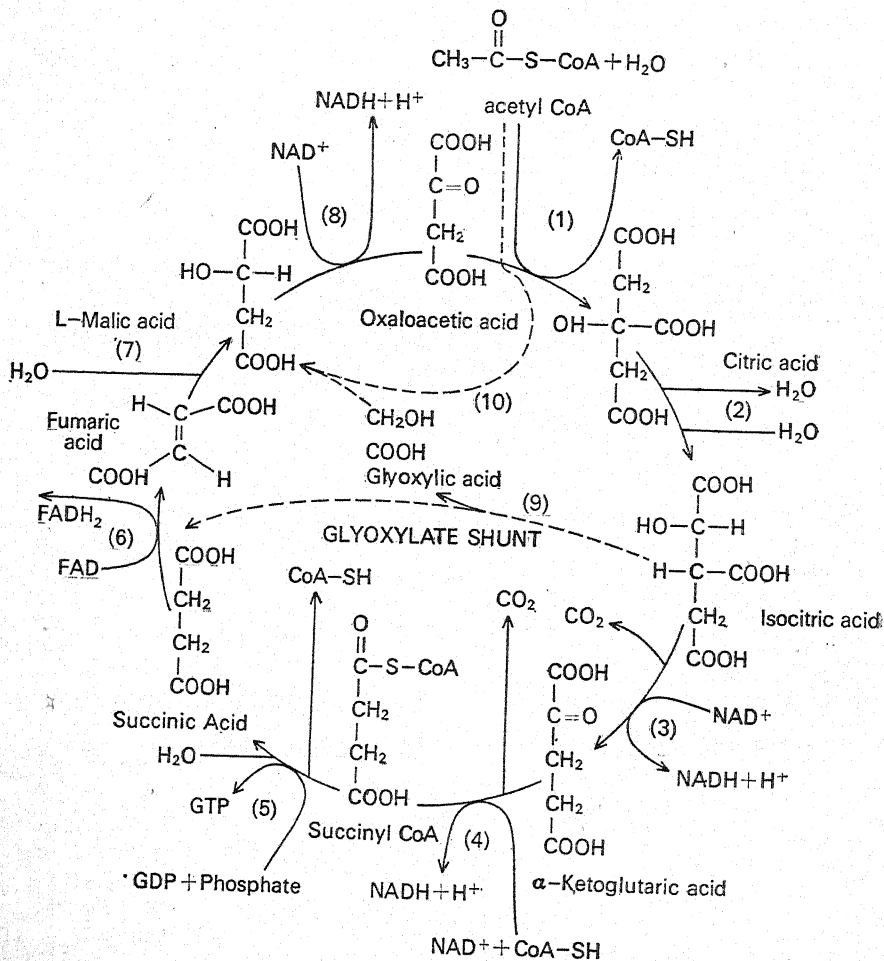


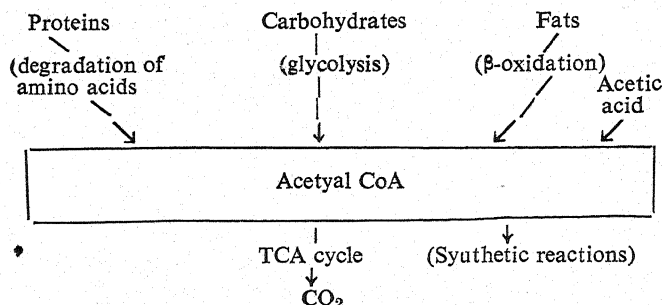
Fig. 8.2. The TCA cycle as currently conceived (1) to (8) different enzymes.

### Steps of the TCA Cycle

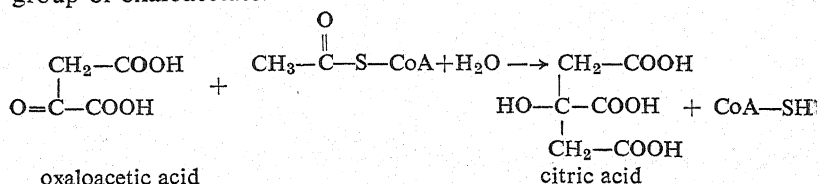
(1) The TCA cycle is initiated with the formation of citric acid, which is a tricarboxylic acid, and hence the name citric acid or tricarboxylic acid cycle. In this step, a condensation reaction takes place between oxaloacetic acid and the acetyl CoA. It must be noted that the acetyl CoA is not only a product of carbohydrate catabolism (via glycolysis and oxidative decarboxylation of pyruvic acid), but it is



formed in various other catabolic reactions of the cell involving a great variety of compounds like protein, fats etc. as shown below.



Nevertheless the most important donor of acetyl group ( $\text{CH}_3\text{CO}-$ ) is pyruvic acid and the most important acceptor is oxaloacetic acid. The condensation reaction between acetyl CoA and oxaloacetic acid is catalyzed by the condensing enzyme citrate synthetase. The interesting and unusual feature of the reaction is that it is the methyl group of acetate and not the carboxyl group, which reacts with the carboxyl group of oxaloacetate:



This reaction can be catalyzed in the reverse direction by a different enzyme citrate lyase to produce oxaloacetic acid and acetyl CoA, in presence of ATP.

The condensing enzyme citrate synthetase was perhaps the first enzyme of the TCA cycle to be obtained in crystalline form from animal cells (Ochoa *et al.* 1951). Among the fungi, partially purified counterparts have been reported from cell-free extracts of yeast (Novelli and Lipmann, 1950) as well as *Aspergillus niger* (Ramakrishnan and Martin, 1955). The fungal condensing enzyme, however, differed from its animal counterpart atleast in one characteristic that it did not require magnesium as its co-factor. Ochoa *et al.* (1951) have also reported the presence of this enzyme in yeast, and it seems that it is well distributed among fungi.

(II) Citrate is converted into isocitrate in the next step, under the catalytic influence of aconitase. According to an earlier concept an

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unsaturated intermediate cis-aconitic acid was formed, which was later converted to isocitric acid, both the steps occurring under the influence of the same enzyme, aconitase. However, it is now believed that the true intermediate remains bound to the enzyme, a fact supported by formation of very little amount of cis-aconitic acid during the operation of the cycle.

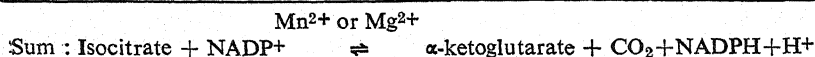
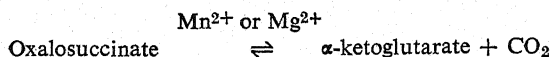
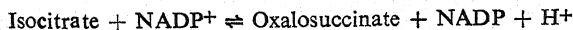
The enzyme aconitase has not yet been obtained in crystalline form, although partially purified products from both animal (Anfin- sen, 1955) and fungal (*Penicillium purpurogenum*, *Aspergillus niger* and *Saccharomyces cerevisiae*: Rahatekar and Raghavendra Rao, 1963) sources have been obtained and they exhibit similar co-factor requirements. Aconitase from *A. niger*, however (Ramakrishnan, 1954; Neilson, 1955), differs from its animal counterpart in being separable by conventional methods into two fractions, viz. (i) one associated with a high molecular weight protein and (ii) the other with a low molecular weight protein. Moreover, the two components also differ in their activities the former, designated as aconitic hydrase (Neilson, 1956) primarily catalyzes the formation of citrate only, while the latter produces both citrate and isocitrate from cis-aconitic acid. The aconitase produced by *Penicillium chrysogenum* resembles the aconitic hydrase component (Neilson, 1962), while that obtained from animal tissues appears to be similar to the other component.

(III) The isocitric acid then undergoes an oxidative decarboxylation under the influence of the enzyme isocitric dehydrogenase to produce  $\alpha$ -Ketoglutaric acid. The reaction has two different phases, one involving oxidation (-2H) and the other decarboxylation (-CO<sub>2</sub>), but both the phases are catalysed by the same enzyme.

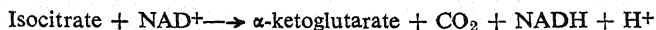
The enzyme isocitric dehydrogenase is different from all other TCA cycle enzymes (except malic dehydrogenase) in that it may also occur in soluble form in the cytoplasm outside the mitochondria. Besides, it exhibits affinities for different coenzymes, and its various types have been reported in yeast (Kornberg and Pricer, 1951) and *Aspergillus niger* (Ramakrishnan and Martin, 1955). One kind of isocitric dehydrogenase exhibit specificity for the co-enzyme NADP<sup>+</sup> while the other type for NAD<sup>+</sup>. The NADP- linked isocitric dehydrogenase appears to be widely distributed among fungi (Barron and Ghiretti, 1953) and has also been partially purified from various microorganisms (Kornberg and Pricer, 1951; Barban and Ajl, 1952; Ramakrishnan and Martin, 1955; Agosin and Weinback, 1956; and Goldman, 1956). The NAD- linked counterpart, on the other hand, has been purified from various animal sources (Plant and Sung, 1954),

and partially separated from the NADP-linked enzyme in yeast (Kornberg and Pricer, 1951) and *A. niger* (Ramakrishnan and Martin, 1955). The reaction catalyzed by these two enzymes also differ, as shown below:

(A) *NADP-linked isocitric dehydrogenase activity.*



(B) *NAD-linked isocitric dehydrogenase activity*



While the NADP-linked isocitric dehydrogenase purified from yeast (Kornberg and Pricer, 1951) catalyzes the decarboxylation of oxalosuccinate, the reductive carboxylation of  $\alpha$ -Ketoglutarate, and the reduction of oxalosuccinate; the latter two activities have not been recorded for the NAD-specific counterpart isolated either from yeast or from heart-tissue (Kornberg and Pricer, 1951). In addition to these, there are still other features which distinguish these two dehydrogenases and it seems that two different enzymes are involved, of which the NADP-linked enzyme is largely distributed in the cytoplasm while the NAD-specific enzyme seems to be mitochondria-bound. The present evidences also indicate that it is the NADP-linked isocitric dehydrogenase which is functional in many fungi.

The reaction catalyzed by this enzyme is significant because it represents the first step towards the degradation of the pyruvate-residue (or its equivalent) through this cycle, during which one of the carbon atom is disposed of as  $\text{CO}_2$ . Also, this is the first reaction of the cycle producing reduced co-enzyme NADPH or NADH.

(IV) and (V). The remaining carbon of the pyruvate-residue (or its equivalent) is disposed of in the very next step, involving the oxidative decarboxylation of  $\alpha$ -ketoglutaric acid. The reaction leading to the conversion of  $\alpha$ -ketoglutarate to succinate is a complex one and follows a sequence (Fig. 8.3) analogous to the oxidative decarboxylation of pyruvic acid. It requires the catalytic action of a multienzyme system ( $\alpha$ -Ketoglutaric dehydrogenase system) comprising thiamine pyrophosphate,  $\alpha$ -lipoic acid, coenzyme A,  $\text{NAD}^+$  and perhaps other yet unidentified co-factors. The reaction sequence may be summarised

(Sandi *et al.* 1956) as under:

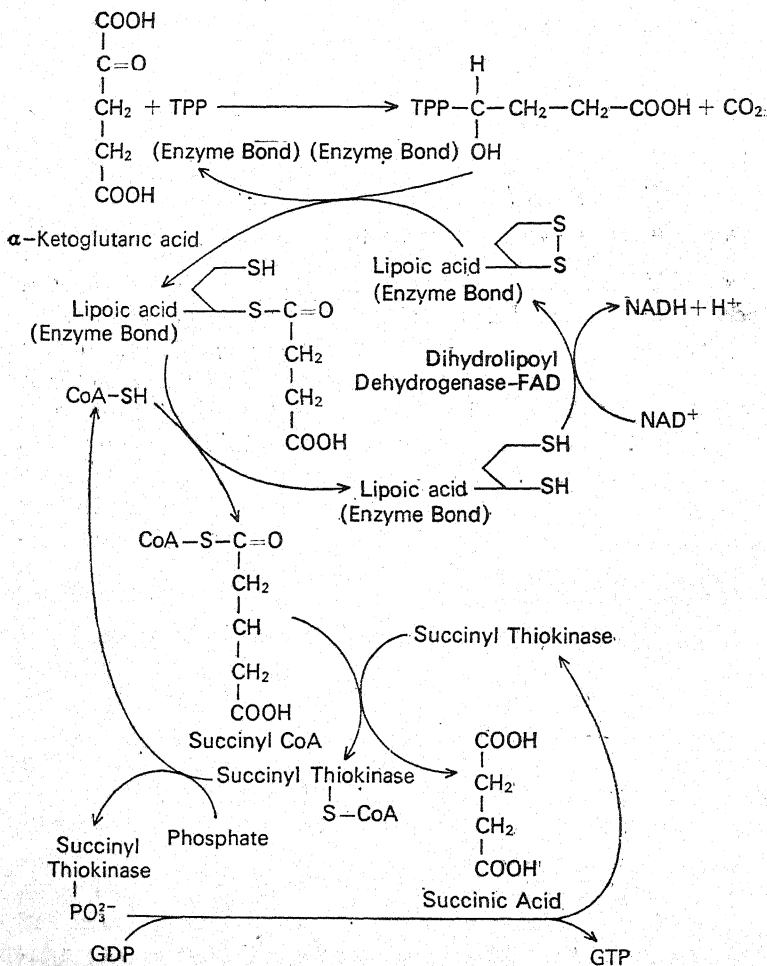
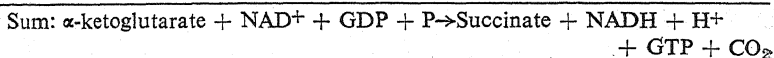
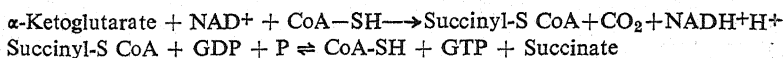


Fig. 8.3. Oxidative decarboxylation of  $\alpha$ -Ketoglutaris.

As shown above, product of the first reaction is the thioester succinyl CoA, which utilizes a part of the energy released in the oxidative decarboxylation. Later this energy is transferred to guanosine diphosphate (GDP) which takes up a phosphate group to form

guanosine triphosphate (GTP). The GTP may directly be utilized by the cell or converted to ATP under the influence of the enzyme nucleoside diphosphokinase:



In this way, altogether four high-energy phosphate groups are produced during this sequence of reaction, three coming from the re-oxidation of the coenzyme NADH reduced in this step, and one as indicated in the above equation.

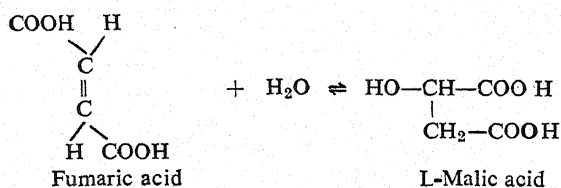
The role played by lipoic acid in this sequence has proved very helpful, as far as studies relating to the oxidation of  $\alpha$ -ketoacid in fungi is concerned. With the use of arsenite as inhibitor, accumulation of  $\alpha$ -ketoacid has been easily demonstrated and arsenite-sensitive respiratory system has been concluded in a variety of fungi (Pickett and Chifton, 1943; Hockenull *et al.*, 1951; Walker *et al.*, 1951; Shu *et al.*, 1954; Chattaway and Thompson, 1956; Goldschmidt *et al.*, 1956; Bonner and Machlis, 1957; Ramachandran and Gottlieb, 1963). However, comparatively little is known about the details of  $\alpha$ -ketoglutaric dehydrogenase system in fungi. The few reports in this regard include those of Ramakrishnan (1954) for *Aspergillus niger* and Holzer *et al.*, (1963) for bakers' yeast. The soluble  $\alpha$ -ketoglutaric oxidase system obtained from yeast mitochondria exhibited co-factor requirements for NAD<sup>+</sup>, coenzyme A, and thiamine pyrophosphate, and was arsenite-sensitive as well.

(VI) This step of the cycle leads to the oxidation of succinic acid into fumaric acid under the catalytic influence of the enzyme succinic dehydrogenase. This enzyme is a flavoprotein and its co-enzyme, flavin adenine dinucleotide (FAD) is covalently bound to it. Obviously NAD<sup>+</sup> has no role to play in this reaction of the cycle. Secondly, the succinic dehydrogenase remains associated with the inner membrane of the mitochondria and, therefore, it is able to cause a direct passage of electrons to the electron-transport chain (discussed in next chapter) and thus it may bypass the NAD<sup>+</sup>-cytochrome reductase section of the chain. The electrons derived from succinate oxidation thus travel only a part of the electron-transport chain and hence it leads to the formation of only two molecules of ATP instead of the normal three.

The enzyme succinic dehydrogenase appears to be the most thoroughly investigated of all the TCA cycle enzymes. Most of the informations have been furnished by Singer and his associates (Singer *et al.*, 1957; Singer and Kearney, 1963), but reports of its partial purification have come from a number of other workers also. Partly

purified succinic dehydrogenase has been obtained from crude extracts of *Neurospora crassa* (Shepherd, 1951) *Aspergillus niger* (Martin, 1954), *Penicillium chrysogenum* (Godzeski and Stone, 1955) and *Myrothecium verrucaria* (Hilton and Smith, 1959). Comparatively, a much more purified succinic dehydrogenase has been obtained from *Claviceps purpurea* (McDonald *et al.*, 1963), possibly due to its more stable nature. The two samples have different pH-optima, viz. the enzyme obtained from *C. purpurea* has an optimum pH at 7.7, while the one from other fungi at pH 6.6, although both are inhibited by malonate.

(VII) The enzyme fumarase catalyzes the next step of the cycle, during which a water-molecule is added to fumaric acid through a reversible hydration reaction as follows:

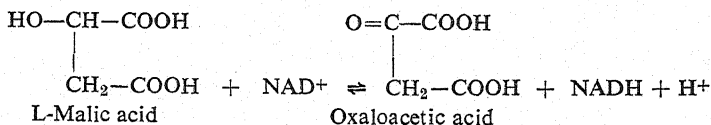


The reaction is stereospecific, as the product of the reaction is always L-malic acid.

The enzyme fumarase has been purified and crystallized from heart muscle tissue of pig (Massey, 1952), although partially purified preparations have also been obtained from *Aspergillus niger* (Ramakrishnan, 1954) and yeast (Favelukes and Stoppani, 1958). Later, fumarase obtained from *Candida utilis* (Hayman and Alberty, 1961) has been shown to comprise two molecular forms, differing in their pH optima, electric charge,  $K_m$  values, as well as acid-ionization constants. However, it is not yet known whether both these forms of fumarase occur in the same yeast cell. The yeast fumarase differs from its mammalian counterpart in all the characteristics enumerated above as well as in its sensitivity to low levels of sulphhydryl inhibitors, and requirement of thiol group for its activity.

(VIII) In this step, malic acid is transformed into oxaloacetic acid, which is the starting material for the TCA cycle. Therefore, this step marks the end of a complete turn of the cycle. This step is yet another oxidative reaction involving the enzyme malic dehydrogenase and  $\text{NAD}^+$ . The dehydrogenation reaction in this step seems to be a single step phenomenon, without any decarboxylative step, but Kun (1963) has indicated the possibilities of both simple and decarboxylative dehydrogenation of malic acid. The former reaction may be re-

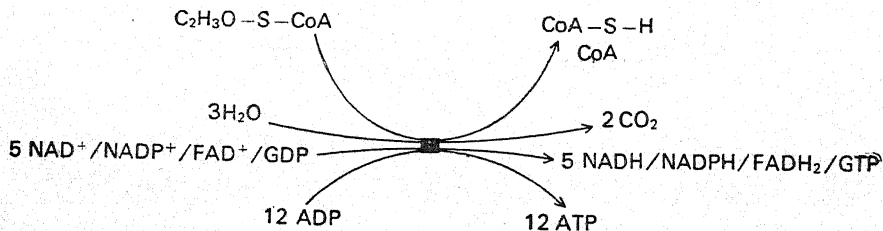
presented as below:



It is a freely reversible reaction, but formation of oxaloacetic acid predominates in the presence of acetyl CoA, which converts the former into citric acid and thus shifts the balance of the reaction towards oxaloacetic acid.

The enzyme malic dehydrogenase has been purified from diverse sources, including cell-free extract of yeasts (Thorne, 1960), pea-epicotyls (Davies, 1961) and animal tissues (Straub, 1942; Wolfe and Neilands, 1956). The soluble malic dehydrogenase obtained from the yeasts differed in various kinetic criteria from its mitochondrial counterparts obtained from various animal and plant tissues. However, malic dehydrogenase has the lowest molecular weight among all the TCA cycle enzymes, although some recent studies with electrophoretic technique have indicated the occurrence of different molecular forms of this enzyme among fungi also. Such molecular forms of enzymes showing same specificity has been termed as isozymes by Markert and Moller (1952). In *Neurospora crassa*, Tsao (1962) found evidences from electrophoretic data for atleast four different malic dehydrogenases. Similarly, Staples and Stahmann (1963) have reported three isozymes of malic dehydrogenase in extracts obtained from the uredospores of *Uromyces phaseoli* (*U. appendiculatus*). Regarding the specificity also, the earlier concept that only L-malate could serve as its substrate, does not seem to be valid now. Davies and Kun (1957) have found that the enzyme could oxidize a wide range of substrates and actually it may be treated as L-hydroxydicarboxylic acid dehydrogenase.

Thus, the net input and output for each turn of the TCA cycle may be summarised as under:



The steps of the cycle involved in the formation of ATP molecules through terminal oxidation and oxidative phosphorylation, as well as their number at each step is shown in Fig. 8.4. From the account of the total input and out-put of the TCA cycle, it may be noted that through each turn, the cycle loses 2 carbon atoms as  $\text{CO}_2$  and also gets an equivalent amount of carbon as an acetyl fragment. So there is no net gain of carbon. Also, there is always a demand of carbon moieties from the cycle for various biosynthetic reactions of the cell, such as synthesis of amino acids requiring  $\alpha$ -keto acids and formation of porphyrins from succinyl CoA, etc. Such a drainage of carbon fragments may obviously jeopardize the continuance of the TCA cycle and, therefore some ancillary steps do exist, which provide extra carbon fragments so vitally needed for the maintenance of the cycle. In other words, the oxaloacetic acid utilized in the first step

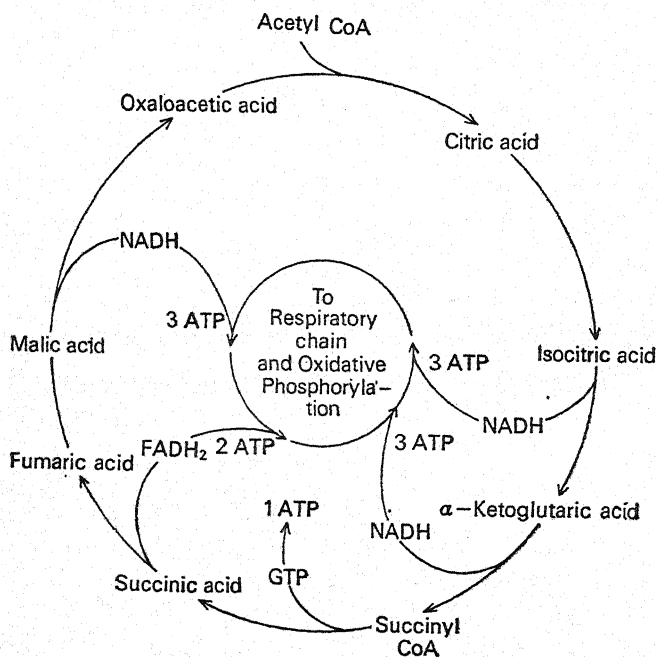
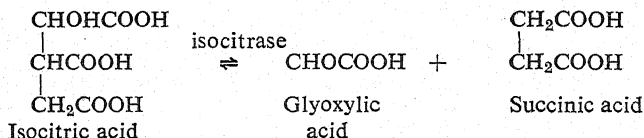


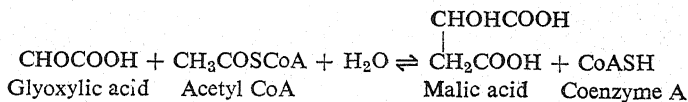
Fig. 8.4. Oxidative steps of the TCA cycle producing reduced Co-enzymes leading to formation of 12 ATP molecules per turn.

of the cycle has to be replenished if the cycle has to continue its operation. Regeneration of oxaloacetic acid through steps other than the TCA cycle may take place in two different ways: (i) through glyoxylate shunt, and (ii) by heterotrophic  $\text{CO}_2$  fixation.

(i) *Glyoxylate shunt or Glyoxylate cycle.* The glyoxylate cycle (Kornberg and Krebs, 1957) and the TCA cycle share quite a number of reactions. The first two steps leading to the formation of isocitrate are common to both the cycles, but thereafter the glyoxylate cycle takes a different route. The decarboxylating stages of the TCA cycle are avoided in the glyoxylate cycle, and the two specific enzymes of this cycle are isocitrase and malate synthetase. The isocitrase brings about a cleavage of the isocitric acid to yield succinate and a two carbon compound glyoxylate:



The enzyme malate synthetase catalyzes the next reaction of the glyoxylate cycle, in which the two carbon compound glyoxylate is condensed with an acetyl CoA, giving rise to malic acid:



The final step of the cycle is similar to that of the TCA cycle and involves the conversion of malic acid to oxaloacetic acid.

Both the enzymes specific to the glyoxylate cycle, *viz.* isocitrase and malate synthetase are now known in many fungi (Kornberg and Collins, 1958; Collins and Kornberg, 1960; Gottlieb and Ramachandra, 1960; Heberling *et al.*, 1960; McCurdy and Cantino, 1960; Frear and Johnson, 1961; Turian, 1961). Besides, partially purified isocitrase (Olson, 1959) and malate synthetase (Dixon *et al.*, 1960) preparations have been obtained from yeast. The enzyme isocitrase is an adaptive enzyme and is not formed in the presence of glucose or succinate in the medium (Krebs and Lowenstein, 1960). Obviously, this nature of the isocitrase allows the glyoxylate cycle to operate only when there is a shortage of succinate.

Thus, the primary role of the glyoxylate cycle appears to supplement the TCA cycle by providing a second point of entry for the acetyl CoA, leading to the synthesis of 4-carbon dicarboxylic acid and helping thereby in the continued operation of the TCA cycle. Another possible role of the glyoxylate cycle seems to provide a mechanism for the assimilation of two carbon compounds like acetate and acetyl CoA, particularly during growth on acetate medium or under abundant production of acetyl CoA. It may be emphasized



here that oxidative decarboxylation of pyruvate into acetyl CoA is in effect an irreversible sequence of reactions and, therefore, the significance of the glyoxylate cycle as a means of acetyl CoA utilization appears to be very high.

(ii) *Heterotrophic CO<sub>2</sub>-fixation*. Carbon dioxide is an essential factor for the growth of microorganisms, including filamentous fungi and yeast. Investigations with diverse fungal forms have led us to believe that assimilation of CO<sub>2</sub> may also be accomplished by heterotrophic organisms like fungi, and is not entirely monopolised by the photosynthetic organisms alone. Heterotrophic CO<sub>2</sub>-fixation is now known in yeast (Ruben and Kamen, 1940), *Rhizopus stolonifer* (Foster *et al.*, 1941), *R. nigricans* (Foster and Davis, 1948), *Candida utilis* (Ehrensward, 1948), *Hansenula anomala*, *Blastocladia pringsheimii*, *Allomyces arbuscula* (Lynch and Calvin, 1952), *Neurospora crassa* (Strauss, 1957), *Aspergillus niger* (Foster *et al.*, 1941; Lewis and Weinhouse, 1951 b; Mosbach *et al.*, 1952), *Puccinia recondita* (Staples and Weinstein, 1959) and *Verticillium albo-atrum* (Hartman and Keen, 1973).

Various routes for heterotrophic CO<sub>2</sub>-fixation have been suggested (Bandurski, 1955; Gunsalus *et al.*, 1955). Some of the routes are schematized below:

1. Phosphoenol pyruvate + ADP + CO<sub>2</sub>  

$$\begin{array}{ccc} & \text{PEP carboxykinase} & \\ & \rightleftharpoons & \\ & \text{Mn}^{2+} & \end{array} \quad \text{Oxaloacetic acid} + \text{ATP}$$
2. Pyruvate + ATP + CO<sub>2</sub>  

$$\begin{array}{ccc} & \text{Pyruvic carboxylase} & \\ & \rightleftharpoons & \\ & & \end{array} \quad \text{Oxaloacetic acid} + \text{ADP} + \text{P}_i$$
3. Phosphoenol pyruvate + CO<sub>2</sub> + H<sub>2</sub>O  

$$\begin{array}{ccc} & \text{PEP carboxylase} & \\ & \rightleftharpoons & \\ & & \end{array} \quad \text{Oxaloacetic acid} + \text{P}_i$$
4. (a) Pyruvate + CO<sub>2</sub> + NADPH  

$$\begin{array}{ccc} & \text{Malic enzyme} & \\ & \rightleftharpoons & \\ & & \end{array} \quad \text{Malic acid} + \text{NADP}^+$$

(b) Malic acid + NAD<sup>+</sup>  

$$\begin{array}{ccc} & \text{malic dehydrogenase} & \\ & \rightleftharpoons & \\ & & \end{array} \quad \text{oxaloacetic acid} + \text{NADH} + \text{H}^+$$

However, definite informations regarding their operation in fungi are available only for the first two routes and most of the data have been furnished by Stoppani and his associates. Their studies on yeast indicated that phosphoenol pyruvate possibly undergoes a  $\beta$ -carboxylation (Stoppani *et al.*, 1958) to give rise to oxaloacetic acid as the primary product (Stoppani *et al.*, 1957) of CO<sub>2</sub>-fixation. Cannata and Stoppani (1959, 1963 a, b) were able to purify a phos-

phosphopyruvate carboxylase from yeast. This enzyme catalyzes the carboxylation of phosphoenol pyruvate in the presence of  $\text{CO}_2$ , ADP and  $\text{Mn}^{2+}$ —forming oxaloacetic acid and ATP. Since this enzyme causes carboxylation and phosphate transfer simultaneously, in recent references the same is referred as phosphoenol pyruvate carboxykinase (PEP carboxykinase). Similar enzyme systems have been reported in *Aspergillus niger* (Woronick and Johnson, 1960) *Neurospora crassa* (Flavell and Fincham, 1968) and *Verticillium albo-atrum* (Hartman and Keen, 1973). Identical mechanism of  $\text{CO}_2$ -fixation was suggested by Staples and Weinstein (1959) in uredospores of *Puccinia recondita* and *Uromyces appendiculatus*. However, further investigations are needed on the distribution of this enzyme in fungi for a more valid estimate of the occurrence of this mechanism of  $\text{CO}_2$ -fixation in fungi.

The only other route of  $\text{CO}_2$ -fixation which has been studied in fungi (although to a very limited extent) is catalyzed by the enzyme pyruvic carboxylase. This enzyme activity was first observed by Woronick and Johnson (1960) in the mycelium of *A. niger*. The enzyme catalyzes the carboxylation of pyruvate, utilizing the energy of ATP. Bloom and Johnson (1962) later found that pyruvate carboxylase of *A. niger* converted pyruvic acid, ATP and  $\text{CO}_2$  into oxaloacetate, ADP and inorganic phosphate (eq. 2). Since then, presence of this enzyme has been recorded only in two more fungi, viz. *Penicillium camemberti* (Stan and Schormuller, 1968) and *Verticillium albo-atrum* (Hartman and Keen, 1973). Necessity for further studies in this regard is more than obvious.

Occurrence of  $\text{CO}_2$ -fixation has also been reported in Mucoraceae (Foster *et al.*, 1941) and *Allomyces arbuscula* (Lynch and Calvin, 1952), but its route in these fungi is not known. Regarding the other two pathways of  $\text{CO}_2$ -fixation very little is known of their occurrence as well as of the concerned enzymes, in fungi. The pathway catalyzed by PEP carboxylase utilizes phosphoenol pyruvate as the substrate, which with its high energy phosphate bond is able to drive the reaction by itself.  $\text{CO}_2$ -fixation, via the last shown route (eq. 4) takes place in two steps, under the influence of two different enzymes as well as co-enzymes.

### OCCURRENCE OF TCA CYCLE IN FUNGI

The presence of a functional TCA cycle in fungi has long been a subject of controversy and in fact the matter has only recently been settled after some comprehensive studies on different aspects of the

problem. Niederpruem (1965) has excellently reviewed the development of the concept of an operative citric acid cycle in fungi and only a brief treatment of this aspect is needed here.

The problem has been investigated both in intact cells as well as cell-free extracts of fungi, at different levels. Accumulation of citric acid in the culture solutions of many fungi, specially *Aspergilli* and *Penicillia* was long recognised as an indirect evidence for existence of a TCA cycle in fungi. Similarly, production of other TCA cycle organic acids, their utilization as sole carbon sources by several yeasts (Barnett and Kornberg, 1960) and their remedial activity against the inhibitors, like iodoacetate on the growth and respiration of *Neurospora crassa* (Ryan *et al.*, 1944) were some of the observations that amply supported the concept.

Physiological studies on respiration in intact cells, more or less under a similar set-up to that applied successfully in animal tissues, could not produce the desired definitive proofs in fungi. The obvious hinderances were due to endogenous respiration in fungi and their ability to assimilate various exogenous substrates. Similarly, the permeability barriers exhibited by fungal mycelium against various TCA cycle intermediates had led to erroneous conclusions and raised serious doubts regarding the significance of these intermediates in normal respiration (Leonard, 1949; Levine and Novak, 1950; Al-Doory, 1959; Garrison, 1961; Newcomb and Jennison, 1962). Likewise, a host of workers reported incomplete substrate activity of various TCA cycle intermediates (Levine and Novak, 1950; Hirsch, 1952; Hockenhull *et al.*, 1954; Krebs, 1954; Bacila *et al.*, 1955; Garrison, 1961). However, these intriguing observations were soon explained by the finding that the same intermediates were metabolised by cell-free extracts of fungi. With this observation the role of permeability barriers in the metabolism of organic acids by fungi was realised (Barron and Ghiretti, 1953; Clark and Wallace, 1958; Litchfield and Ordal, 1958). Since then different techniques have been employed to overcome such permeability barriers and studies with *Schizophyllum commune* (Wessels, 1959), baker's yeast (Kovac, 1961) etc. have helped sufficiently to establish the concept of a functional TCA cycle in fungi.

The problem of permeability barrier was also confronted in studies with respiratory poisons in fungi, and inconsistent results were obtained with different fungi, atleast in case of malonic acid, monofluoroacetic acid, etc. Malonic acid, which produced strong inhibitory effect on respiring cells of *Rhodotorula gracilis* (Litchfield and Ordal, 1958), failed to arrest respiration in the cells of *Merulius niveus*,

*Rhizopus nigricans* (*R. stolonifer*) (Barron and Ghiretti, 1953) and *Pullularia pullulans* (Clark and Wallace, 1958). That such contrasting behaviour of malonate is also due to permeability barrier, was indicated from experiments with cell-free extract. Since then, malonate has been reported to be an effective inhibitor of succinic dehydrogenase present in the cell-free extracts of many fungi (Clark and Wallace, 1958; Hilton and Smith, 1959; Chattaway *et al.*, 1960).

Similar permeability barrier to monofluoroacetate has been reported in *Penicillium chrysogenum* (Goldschmidt *et al.*, 1956), although in *Neurospora crassa* (Strauss, 1955) and yeast (Kalnitsky and Barron, 1947) it causes significant inhibition. The primary contribution of these studies, particularly the concept of permeability barriers, has been a contradiction of the earlier idea that a functional TCA cycle is lacking among fungi.

Evidences obtained from experiments with isotopic labelled glucose and acetate were also not conclusive. However, the difficulty was due to an additional and independent mechanism of acetate oxidation in fungi, which we now know as glyoxylate cycle. Otherwise, the content and distribution of radioactivity in the citric acid produced by baker's yeast, (Weinhouse and Millington, 1947), was suggestive of a functional TCA cycle.

Another line of investigation pertaining to this aspect, employed cell-free extract in which the presence and location of different TCA cycle enzymes were studied. Mitochondria, the seat of all but two TCA cycle enzymes received considerable attention. Although, respiratory granules in yeasts (Lindegren, 1949), Mudd *et al.*, 1951; Mundkur, 1953; Hartman and Liu, 1954; Bautz, 1955; Williams *et al.* 1956) and in a variety of filamentous fungi (Moore and McAlear, 1963; Lindenmayer, 1955) have been adequately recorded on the basis of cytochemical as well as ultra structural studies of mitochondria, attempts to study the subcellular localization of the TCA cycle enzymes met with limited success. However, the problem was largely of methodology and was soon overcome. Nossal (1954 a) used a highly rapid mechanical disintegrator to prepare particulate fractions from yeast, which could oxidize all the intermediates of TCA cycle, with the concomitant consumption of molecular oxygen. With still more efficient technique as well as improved isolation media, Linnane and Still (1955) isolated highly active respiring mitochondria from yeast, which could oxidize the principal intermediates of the TCA cycle. Particulate matters with various TCA cycle enzymes have also been separated from *Neurospora crassa* (Haskins *et al.*,

1953), *Allomyces macrogynus* (Bonner and Machlis, 1957), *Candida albicans* (Nozu *et al.*, 1958), *Fusarium lini* (Kikuchi and Barron, 1959), *Aspergillus oryzae* (Imamoto *et al.*, 1959), *Myrothecium verrucaria* (Hilton and Smith, 1959), *Schizophyllum commune* (Wessels, 1959; Niederpruem and Hackett, 1961), *Puccinia graminis* (White and Ledingham, 1961), *Fusarium oxysporum* (Maruyama and Alexander, 1962), and other fungi, (Dowler *et al.*, 1963). Further, role of these respiratory granules in oxidative phosphorylation in yeast (Hodges and Marx, 1959; Vitols and Linnane, 1961) as well as purification of electron transport particles, also from yeast (Mackler *et al.* 1962) have almost established that the respiratory granules of yeast and filamentous fungi perform essentially the same function as do the mitochondria of plants and animals.

All these evidences (although not too many) along with informations regarding different TCA cycle enzymes (as already discussed) amply demonstrate that the occurrence of a functional TCA cycle among fungi is now established.

### ROLE OF TCA CYCLE IN FUNGI

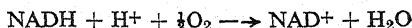
TCA cycle is an amphibolic pathway (Davis, 1961), which functions both in the catabolic generation of ATP and in the anabolic synthesis of important biosynthetic precursors. Originally, it was thought to be a means for terminal oxidation of carbohydrates, but its role as a donor of carbon moities for amino acid synthesis by transamination and reductive amination is now well recognised. The major biosynthetic products are derived from  $\alpha$ -ketoglutaric and oxaloacetic acids. The other important product of this cycle is succinyl CoA which is a precursor of both methionine and porphyrins. The amino acids synthesized from TCA cycle precursors contribute towards the synthesis of proteins, purines and pyrimidines, and the TCA cycle thus provides a link between carbohydrate and nitrogen metabolism. Ehrensvar *et al.* (1951) with isotopic tracer technique demonstrated that *Candida utilis* synthesized aspartic acid with the labelled carbon skeleton supplied as acetate. Similar results were recorded by Krebs *et al.* (1952) and Wang *et al.* (1953) for glutamic acid synthesis in yeast, who concluded that the primary role of TCA cycle in fungi is to furnish carbon moities for biosynthetic reactions. Further studies with *Candida utilis*, *Neurospora crassa* (Abelson *et al.*, 1953), Anderson-Kotto *et al.*, 1954; Abelson and Vogel, 1955) and *Zygorhynchus moelleri* (Moses, 1957) have not only confirmed that aspartic and

glutamic acids were derived from TCA cycle compounds, but have shown that the contribution of the TCA cycle can be traced further into many other amino acids, which are obviously derived from aspartic and/or glutamic acids. Another significant role of the TCA cycle is the coupling of dehydrogenase systems with the respiratory chain. Moreover, the cycle in association with the heterotrophic  $\text{CO}_2$ -fixation also serves to replenish the cell carbon.

## RESPIRATION—III

## TERMINAL OXIDATION

The reactions of TCA cycle described in the preceding chapter are highly aerobic, and proceed only if oxygen is available to the cell. This is because, the functioning of the TCA cycle depends upon the continuous availability of the reoxidized  $\text{NAD}^+$  and succinic dehydrogenase as well as their dehydrogenating activity at various steps. The reduced  $\text{NADH}$  and  $\text{FADH}_2$  (the active group of succinic dehydrogenase) produced at four different steps of the TCA cycle are reoxidized at the expense of the dissolved oxygen in the following manner:



However this is just to represent the overall process, which actually involves at least half a dozen hydrogen-transfers through the activity of a chain of oxido-reductive enzymes, designated as the respiratory oxidation chain. Oxidation chains may be different in different cells, or even in the same cell, more than one type may operate. However, the present state of our knowledge indicates a wide occurrence of at least one such series, which is thought to perform the major oxidative function during aerobic respiration.

In the year 1886 MacMunn concluded that a wide range of biological materials contained iron-containing compounds, the haems, which on being reduced gave characteristic visible absorption spectra, but they disappeared as the haems were reoxidized. He proposed that the haems had some respiratory function. MacMunn's hypothesis was supported many years later by Keilin (1925) who recorded the presence of haems in yeast, animal cells and non-green plant tissues. He designated such compounds as cytochromes, and recognised its three kinds, viz. cytochrome a, b and c, on the basis of their differential absorption spectra. However, many more kinds of cytochromes with widespread distribution among diverse living organisms are

known today. Obviously, it is now generally accepted that a respiratory chain composed of cytochromes (plus a few other carriers) is the most common series of enzymes functioning in respiration in a large number of organisms. Cytochromes have been identified in all groups of fungi (Boulter and Derbyshire, 1957), and different kinds of cytochromes have been isolated and purified from different species of fungi. However, most of the detailed work has been confined to a single species of yeast, viz. *Saccharomyces cerevisiae* and among the filamentous fungi only *Aspergilli*, *Penicillia* and *Neurospora* have been studied to any considerable extent. Otherwise the rest of the members have practically been left untouched. Moreover, the sequence of the cytochromes forming the respiratory chain is not completely known even for a single fungal species. Although it has often been claimed that the cytochrome system of fungi is strikingly identical to those operative in mammalian and avian cells (Lindemeyer, 1965), yet efforts to substantiate this possibility as well as to elucidate the actual sequence of their operation in different fungi are very much required.

### Electron Transport and Respiratory Chain

The general sequence of electron transport from NADH to molecular oxygen, according to the present concept, and based on the standard redox-potential of the various cytochromes as well as on the basis of data obtained from certain inhibitor studies is represented.

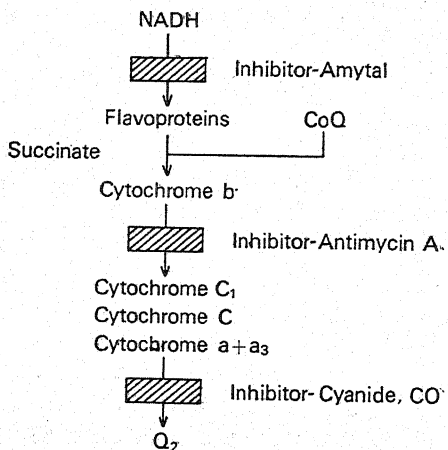


Fig. 9.1. Respiratory-chain, (based on redox-potential of the components) and the sites of action of inhibitors.



in Fig. 9.1.

Experiments with respiratory inhibitors have yielded data generally in support of the electron-transport chain (Fig. 9.1). It has been observed that three different points of the chain are attacked specifically by these inhibitors. When the aerobic respiration rate is optimum, the reduced NADH is continuously available for oxidation through the electron transport chain, and the different electron carriers are in varying states of oxidation. However, it has been observed that addition of dilute cyanide leaves all the carriers in fully reduced condition, which is obviously due to the inhibition of  $a_3$  component of the cytochrome  $a+a_3$ . Similarly, when antimycin A is added, NADH, flavoprotein and cytochrome  $b$  become reduced, while cytochrome  $c$  and  $a+a_3$  get completely oxidized. Sodium amobarbital makes only NADH fully reduced. These observations support the sequence of the carrier enzymes as shown in Fig. 9.1 but it may be a simple or branched chain, and in fact cytochrome  $b$  has been considered to occupy a side position.

As shown in Fig. 9.1 the electrons from reduced co-enzyme NADH are passed on to an oxidized flavoprotein, which transfers them to a series of cytochromes probably through a ubiquinone (CoQ). The sequence of different cytochromes, viz. cytochrome  $b$  and  $c_1$  as well as the position of ubiquinone also is not yet fully resolved. Therefore, what component of the chain receives the electrons donated by the flavoprotein and through what exact sequence of carriers they travel upto cytochrome  $C$  is not quite clear. The nature of the chain beyond cytochrome  $C$  is generally known and once the electrons are accepted by cytochrome  $C$ , they are sent to the cytochrome  $a+a_3$  complex, which finally transport them to the molecular oxygen, and hence called cytochrome oxidase.

As indicated above the final step may be inhibited by cyanide. But, there have been several reports of cyanide insensitive electron carrier systems in different fungi, including those in *Myrothecium verrucaria* (Darby and Goddard, 1950), *Ustilago sphaerogena* (Grimm and Allen, 1954), *Candida albicans* (Ward and Nickerson, 1958), etc. These reports are suggestive of operation of another electron transport system in these fungi. Beevers (1961) suggested that this may be due to the presence of a flavoprotein oxidase. However, the occurrence of a specific cytochrome among these fungi, which may be insensitive to cyanide seems more probable, especially because such a cytochrome ( $b_7$ ) is known in higher plants, and is active under conditions inhibiting for the functioning of normal cytochrome ( $a+a_3$ ).

Nevertheless, cytochrome  $b_7$  has not as yet been demonstrated among fungi and further studies on this aspect may be revealing.

Similarly, there are certain reports, which suggest that some fungi possess antimycin A as well as azide-tolerant electron transport systems. Sherald *et al.* (1972) found that spores of *Ustilago maydis*, and *Ceratocystis ulmi* possessed an alternative pathway of electron transport, which appears to divert electrons to  $O_2$  from some point on the substrate side of antimycin A block. Similar situation was recorded in *Rhodotorula mucilaginosa* by Shin *et al.* (1970). Kawakita and Masao (1970) observed that conidia of *Aspergillus oryzae* germinated both in antimycin A free and antimycin-containing media. The mycelia developing in the drug-free media stopped growing immediately after antimycin A was added. But after several hours, the growth was resumed, which was found to be antimycin A-insensitive. The mycelia developing under these two conditions exhibited difference in sensitivity to the drug. While the mycelium germinating in antimycin A containing medium were insensitive to the drug, those developing in the antimycin A-free medium were sensitive to the drug. The difference was attributed to the different characteristics of the mitochondrial respiratory systems of the two mycelia.

### Respiratory Enzymes

**Flavoproteins.** Flavoproteins are enzymes containing a flavine compound as the co-enzyme. Two types of flavine compounds *viz.* flavine mononucleotide (FMN) or riboflavine phosphate, and flavine dinucleotide (FAD) are known to constitute the prosthetic group of flavo-protein enzymes. The first enzyme of this kind was isolated from yeast by Warburg and Christian (1932), and was called the "old yellow enzyme." The structure and role of its prosthetic group (FMN) was established by Kutin *et al.* (cf. Theorell, 1951).

In respect of electron transport chain, our concern is only with two kinds of flavoproteins, which are found in the mitochondria, *viz.* the one involved in the reoxidation of NADH, and the other, which oxidizes succinate. Little is known of fungal NADH dehydrogenases [NADH : (acceptor) oxidoreductase], although its mammalian counterpart has been studied considerably (Singer *et al.*, 1957 a ; Singer, 1961; Ernster, 1961). Earlier FAD was thought to comprise its prosthetic group but subsequent investigations have suggested it to be FMN (Huennekens *et al.*, 1961; King *et al.*, 1962; Ringler *et al.*, 1963).

FMN has also been implicated as the prosthetic group of a NADPH oxidizing flavoprotein present in yeast (Lindenmayer, 1965). This enzyme catalyzes the oxidation of NADPH by cytochrome c directly.

The succinate dehydrogenase [Succinate: (acceptor) oxidoreductase] on the other hand seems to be characterized by FAD. Singer *et al.* (1957 b) studied and purified the succinic dehydrogenase from yeast and found it to contain FAD and 4 atoms of nonhaem iron per molecule of flavine. However latter investigations have shown that the ratio of iron: flavine may be 1. The enzyme succinate: cytochrome c is, however, lacking when the yeast cells are grown anaerobically (Slonimski, 1956) and is replaced by succinate: phenazine-methosulphate oxidoreductase (Hebb *et al.*, 1959). A soluble succinate: phenazine-methosulphate oxidoreductase was also found in *Claviceps purpurea* (McDonald *et al.*, 1963).

Some other flavoproteinous enzymes including nitrate and nitrite

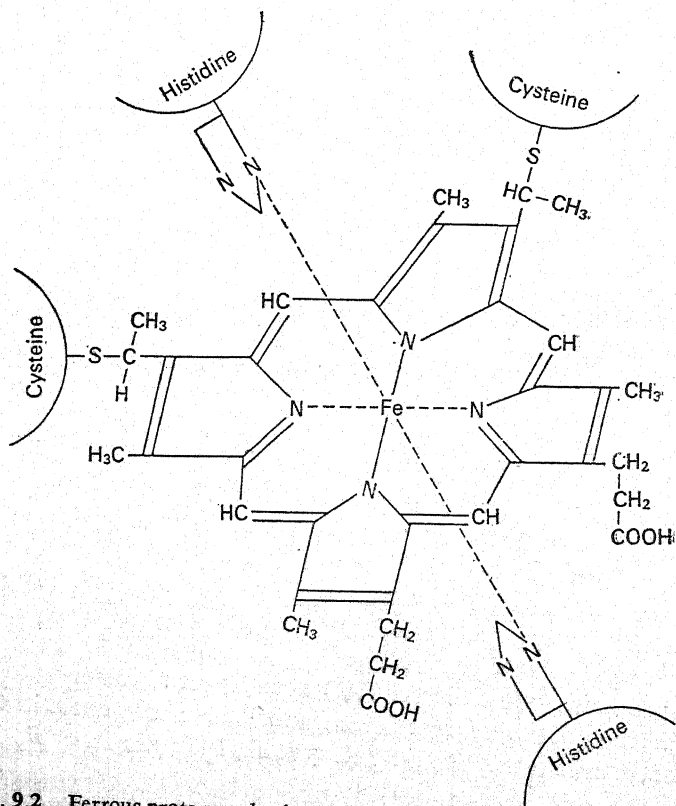


Fig. 9.2. Ferrous proto-porphyrin IX (Haem) as linked with the protein in cytochrome-C.

reductases, various dehydrogenases, glucose and L-amino acid oxidases etc. are known in fungi but they seem to have no function whatsoever in electron-transfer.

*Cytochromes.* These are low molecular weight protein molecules with haems as their prosthetic groups. Cytochromes may be of different types depending upon the nature of their protein-fraction, the haem groups, as well as their binding-pattern. The cytochromes are able to perform their principal biological function with the help of the haem iron, which is characterised by a reversible valency, *i.e.*  $\text{Fe}^{++} \rightleftharpoons \text{Fe}^{+++}$ . The haems are iron-porphyrins, with an atom of iron placed centrally and co-ordinately linked with the nitrogens of the pyrrole rings, as shown above.

The iron porphyrin groups may have different side-chains and they may be differently bounded to the protein, to form various kinds of cytochromes. Although four types of cytochromes *viz.* A, B, C and D types have been distinguished, but the last one has not been found in fungi and is known only in some bacteria. Different cytochromes are denoted by lower-case letters, *e.g.* cytochrome *a*, *a*<sub>3</sub>, *b*, *b*<sub>2</sub>, *c* and *c*<sub>1</sub>. Cytochromes have two distinct absorption spectra, one for the reduced and the other for the oxidized state of the haem-iron, and on this account they differ from all other haemoproteins. Absorption spectra of the principal cytochromes, alongwith some other characteristics are presented in Table 9.1.

TABLE 9.1  
SHOWING CHARACTERISTICS OF SOME PRINCIPLES  
CYTOCHROMES

Cytochrome	Redox potential ( <i>E</i> ' <sub>o</sub> ) in volts	Absorption peaks			Mol. Wt. Monomer	Reaction with		
		$\alpha$	$\beta$	$\gamma$		CO	HCN	O <sub>2</sub>
<i>a</i>	+0.29	603	none	452	66000	—	—	—
<i>a</i> <sub>3</sub>	—	603	none	448	—	+	+	+
<i>b</i>	0.04	563	530	432	28000	—	—	slight
<i>b</i> <sub>3</sub>	0.06	560	529	425	—	—	—	+
<i>b</i> <sub>5</sub>	+0.02	557	527	423	—	—	—	+
<i>b</i> <sub>7</sub>	-0.03	560	529	—	—	—	—	+
<i>c</i>	+0.26	550	521	417	12000	—	—	—
<i>c</i> <sub>1</sub>	+0.26	553	524	418	40000	—	—	—

Since Keilin's (1925) discovery of haems in yeasts, cytochromes have been studied in a large number of fungi and similar absorption-spectra have been recorded for most of them (Keilin and Tissieres, 1953; Grimm and Allen, 1954; Boulter and Derbyshire, 1957;

Kikuchi and Barron, 1959; Niederpruem and Hackett, 1961; Gleason, 1968; Shin *et al.*, 1970). Absorption spectra were originally the only criterion to identify and distinguish the cytochromes, but later on usual methodology of protein-separation was employed and some of the cytochromes were isolated and purified. In some cases their coenzymes and apoenzymes have also been isolated and identified. Cytochrome c is easier to isolate and, therefore, has been studied in greater detail. Its prosthetic group is iron protoporphyrin IX identical with the haem of haemoglobin, and possesses a saturated side-chain. The entire amino acid sequence of yeast cytochrome C has been elucidated by Margoliash *et al.* (cited by Lindenmayer, 1965). Cytochrome c is more resistant to heat and, therefore, its physical and chemical characteristics have been studied in considerable detail. Lindenmayer (1965) has recorded the properties of fungal cytochrome c.

Of the other fungal cytochromes, cytochrome b is the least known both in regard to its form and function. Its prosthetic group, an iron-protoporphyrin, remains attached to the protein by two iron-nitrogen bonds. The cytochrome b is not affected by cyanide or carbon monoxide, and in that way it is similar to cytochrome c, and  $c_1$ . Cytochrome  $c_1$  resembles cytochrome c to a considerable extent and hence was discovered very late among fungi. The limited information on cytochrome  $c_1$  has been furnished by Lindenmayer (1965).

*Cytochrome oxidase.* Cytochrome oxidase refers to that respiratory chain enzyme which makes the final transfer of hydrogen to oxygen itself. There have been some difficulties in its designation. In the beginning this enzyme was referred as the "oxygen transferring enzyme" (Warburg and Negelein, 1934). This characteristic is now known to be possessed by cytochrome  $a_3$  (Keilin and Hartree, 1939), but some investigators consider cytochrome oxidase as a complex of cytochrome a +  $a_3$ , and designate this enzyme complex as cytochrome a (Wainio and Cooperstein, 1956; Okunuki *et al.*, 1958; Green, 1959). However, the two fractions envisaged have not been separated as yet, although the enzyme has been isolated from mitochondria. On the basis of its function, this enzyme is usually denoted as cytochrome oxidase or cytochrome c oxidase (cytochrome c :  $O_2$  oxidoreductase).

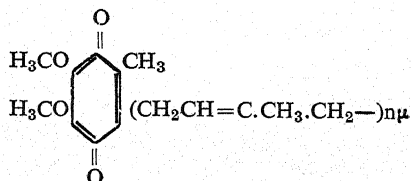
The chief argument in support of presence of two A-types of cytochromes in this complex has been advanced by Keilin and Hartree (1939), who observed that when  $CO$ , cyanide and azide were added, the  $\alpha$  and  $\gamma$  bands at 605 and 445  $m\mu$  exhibited different behaviour. Hence existence of two pigments was suggested: (i) cytochrome a, which does not combine with  $CO$  or other inhibitors and has an

unusually high  $\alpha:\gamma$  band extinction ratio; and (ii) cytochrome  $a_3$  which shows a normal  $\alpha:\gamma$  ratio and is able to combine with CO, cyanide and azide, besides oxygen. In fact cytochrome  $a_3$  differs from all other cytochromes in its reactions with the inhibitors (Table 9.1). Its reaction with carbon monoxide (CO) is of particular interest. CO combines with ferrous iron of the enzyme thus competing with oxygen, and forms a carbonyl which is unstable to light. The inhibition caused is, therefore, removed by strong illumination. Other inhibitors, like HCN, azide, etc., however, combine with the ferric iron and hence they are non-competitive. All these inhibitors react with iron, which is present in the enzyme only in small quantity. Therefore, even small quantities of inhibitors may bring about complete inhibition of the enzyme.

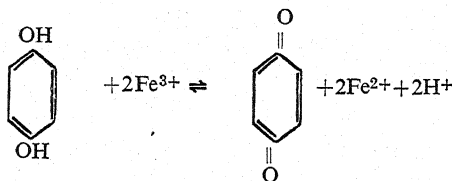
The prosthetic group of cytochrome oxidase seems to be heme a, which is a formyl porphyrin iron compound. In addition, it appears to contain 1 or 2 atoms of copper firmly attached to it (cf. Lemberg, 1961; Paul, 1960). However the role of Cu in oxidation, if any, is far from understood.

Fungal cytochrome oxidases have been studied in several fungi, including *Rhizophlyctis rosea* and *Blastocladiella emersonii* (Cantino and Hyatt, 1953; Horgen and Griffin, 1969), *Allomyces* sp. (Bonner and Machlis, 1957; Turian, 1960), *Aspergillus* spp. (Martin, 1954; Imamoto *et al.* 1959; Iwasa, 1960), *Neurospora* spp. (Shepherd, 1951, Nicholas *et al.* 1954; Owens, 1955; Turian, 1960; Holton, 1960), *Glomerella cingulata* (Sussman and Markert, 1953), *Fusarium lini* (Kikuchi and Barron, 1959), *Schizophyllum commune* (Niederpruem and Hackett, 1961), *Puccinia graminis* (White and Ledingham, 1960), and *Polystictus versicolor* (Boulter and Burges, 1955). Recently, cytochrome oxidases of *Rhodotorula mucilaginosa* (Shin *et al.*, 1970), *Leptomitus lacteus* and *Apodachlya punctata* (Gleason, 1968) have also been investigated.

**Ubiquinones (Coenzyme Q).** These are benzoquinone derivatives with isoprenoid side chains of varying length, and have the following structure:



Its role in electron transport has been studied in detail in systems other than fungi, and the reports on fungal ubiquinones (U.Q.) are mere records of their presence. From analogous evidences, ubiquinones seem to perform the oxidation-reduction activity at the quinol-quinone level:



Evidences for participation of ubiquinones in the terminal oxidation chain are although conjectural have already established its role in electron transport. Some of the evidences are as follows:

(1) Removal of CoQ from mitochondria causes a loss in their ability to oxidise succinic acid.

(2) The lost ability of mitochondria is restored when CoQ is added.

(3) Succinate causes reduction of mitochondrial ubiquinone, which is reoxidized by atmospheric oxygen.

(4) The position of CoQ is indicated by the observation that antimycin A inhibits the oxidation of the internal or exogenously added quinol, and that when NADH is the reductant, the reduction of ubiquinone by mitochondria is blocked by amytal (Fig. 9.1).

(5) Spectrophotometric evidences indicate sufficiently rapid rates of reversible oxidation-reduction of ubiquinones, justifying their inclusion in electron transport chain.

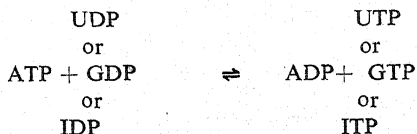
Thus, the part played by ubiquinones as a component of electron transport system is now more or less accepted atleast in mammalian mitochondria (cf. Hatefi, 1963). Among fungi, there are reports regarding the occurrence of UQ in whole cell or mycelia. Fungi in which presence of ubiquinones has been reported include some *Aspergilli* (Packter and Glover, 1960; Lavate *et al.*, 1962), *Penicillium stipitatum*, Lavate *et al.*, 1962), *Absidia corymbifera* (Lester *et al.*, 1958, 1959; Gloor *et al.*, 1958; Lester and Crane, 1959), *Neurospora crassa* (Lavate *et al.*, 1962), *Ustilago maydis* and *Agaricus campestris* (Erickson *et al.*, 1960) as well as some yeasts (Lester and Crane, 1959; Lester *et al.*, 1958, 59; Sugimura, *et al.*, 1964). Different fungi were found to contain slightly different kinds of UQ, the most common types being UQ6, UQ9 and UQ10. Similarly, the same fungus contained different amount, and different types of UQ under aerobic or anaerobic condition.



## OXIDATIVE PHOSPHORYLATION

Glucose possesses 674 Kcal of heat of combustion and the standard free energy change is approximately 686 Kcal. Degradation of each glucose molecule thus releases a large chunk of energy, which if left unutilized will be dissipated as heat energy. Any growing biological system can ill afford such luxury and as such it must possess adequate mechanism to trap and store this energy for subsequent use in their metabolic activities. This is generally achieved through formation of new chemical bonds, which store energy.

A thoroughly investigated system of utilization and storage of respiratory energy is the coupling of the reactions of oxidation with the esterification of inorganic phosphate to form organically bound pyrophosphate. Adenosine diphosphate (ADP) serves as the inorganic phosphate acceptor and is transformed into adenosine triphosphate (ATP). Other pyrimidine phosphates may also take part in such oxidative phosphorylation, or alternatively they may exchange phosphate group from ATP under the influence of an enzyme nucleoside diphosphokinase. Transphosphorylation reactions among different nucleotides may be shown as under:



Such a coupling of oxidation and phosphorylation may occur under aerobic or anaerobic conditions. As for example, the phosphorylation occurring in course of glycolysis (during conversion of phosphoglyceraldehyde into phosphoglyceric acid) do not require aerobic conditions. That phosphorylation is also coupled to aerobic oxidation was first recognised by Kalcker (1937). Soon this observation was confirmed by Belitser and Tsybakova (1939) as well as Needham *et al.* (1941), who also concluded that during oxidative phosphorylation ADP is converted into ATP, and under suitable experimental conditions, more than one phosphate was taken-up for each oxygen atom consumed. In fact, under optimum conditions the phosphate to oxygen ratio (P:O ratio) almost approached 3 for NAD<sup>+</sup>-linked substrates, and 2 for succinate and other flavoprotein dehydrogenases, which reduce the cytochrome region of the chain directly. The only exception seems to be  $\alpha$ -keto-glutarate which gives a P:O ratio approaching 4 due to the formation of GTP from succinyl CoA. The value



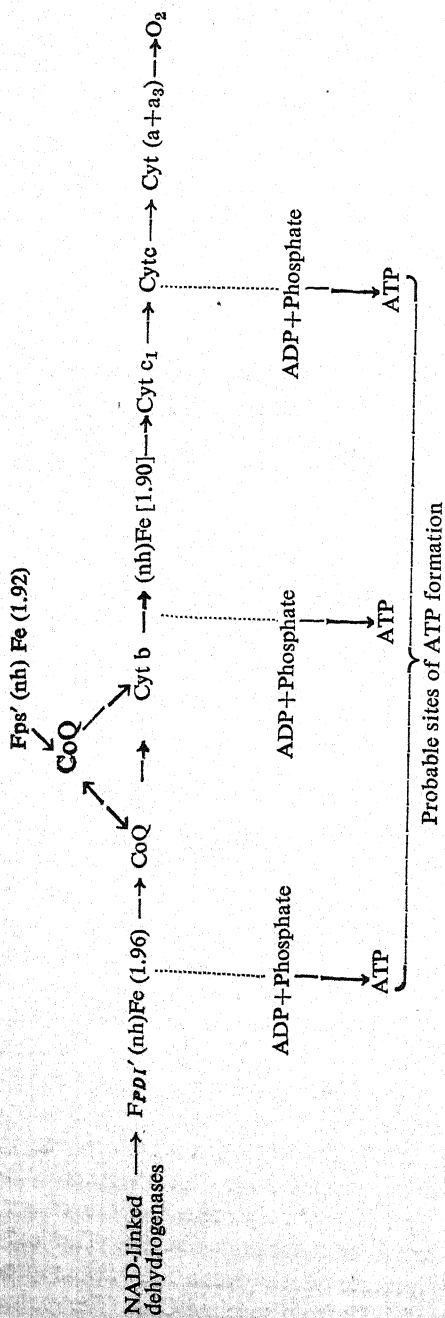


Fig. 9.3. Showing probable sites of phosphorylation on the electron-transport chain.

of P:O ratio higher than one indicates that more than one phosphates are esterified each time a pair of electrons are transferred from NADH to oxygen. It has, therefore, been suggested that three phosphorylative steps occur at three different sites on the electron transport chain. Isotopic data indicate that phosphorylations occurred (i) during electron transfer from NADH to the ubiquinone-cytochrome b portion of the chain, (ii) during passage of electron from cytochrome b to cytochrome c and (iii) during passage of electron from cytochrome c to oxygen (Fig. 9.3).

However, much remains to be understood about the process of phosphorylation itself, although this has been a field of intense investigation for the last several years. Most of the work on this aspect have been confined to animal or bacterial systems, obviously on account of the difficulties inherent in isolation of functional mitochondria of fungi. Appropriate methods of isolation of active mitochondrial particles of fungi were devised rather late (Nossal, 1953; Eddy and Williamson, 1957; Chaix, 1961; Vitols and Linnane, 1961). Consequently, investigations into the oxidative phosphorylation of fungi have lagged behind, and has been studied only in a few forms including yeast (Vitols and Linnane, 1961), *Aspergillus oryzae* (Iwasa, 1960) and *Allomyces macrogynus* (B.A. Bonner and Machlis, 1957). Phosphorylation (P:O) ratio for oxidation of a few substrates by fungal mitochondria are tabulated in Table 9.2.

TABLE 9.2

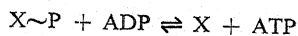
SHOWING P:O RATIO OBSERVED FOR OXIDATION OF  
VARIOUS SUBSTRATES BY FUNGAL MITOCHONDRIA

Organism	Substrate	P:O
Yeast	Succinate	1:6
Yeast	NADH	1:6
<i>Aspergillus oryzae</i>	—	1.6 & less
<i>Allomyces macrogynus</i>	—	Very low

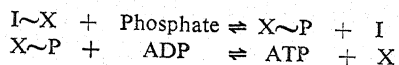
### Mechanism of Phosphorylation

Fungi seem to have contributed very little in the understanding of the mechanism involved in oxidative phosphorylation. Most of the data have been furnished by studies on animal mitochondrial systems. Various hypotheses have been proposed to explain the linkage between oxidation and phosphorylation, as well as the control of ATP-synthesis.

It has been proposed that during synthesis of ATP from ADP either (through transphosphorylation from pre-existing ATP molecules) or from inorganic phosphate, some intermediate compound takes part in the transfer of phosphate group. An unidentified intermediate (X) may participate in transphosphorylation in the following manner:

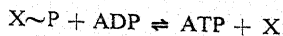
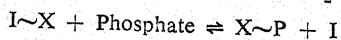
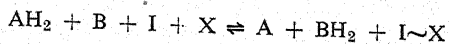


The X intermediate gets phosphorylated ( $X\sim P$ ) in the presence of ATP, when the reaction proceeds towards the left, and the same is used to phosphorylate an ADP molecule, when the reaction proceeds to the right. Another intermediate compound I is thought to participate during phosphorylation involving inorganic phosphate. The overall process involves two steps and two hypothetical intermediates, I and X:



Some other activities including ATPase activity and exchange of the oxygen atom of water with one of the oxygens of inorganic phosphate, have also been found to be associated with oxidative phosphorylation, and together they all remain linked to the inner membrane of the mitochondria.

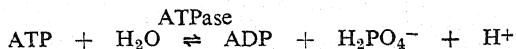
The various hypotheses regarding mechanism of oxidative phosphorylation include (i) chemical coupling hypothesis, and (ii) chemiosmotic hypothesis. According to the chemical hypothesis, which is the oldest and the most accepted one, envisages that oxidative phosphorylation does not differ basically from substrate-level phosphorylation. The general mechanism suggested is:



where A and B are two consecutive carriers of the electron transport chain in oxidized form, and  $AH_2$  and  $BH_2$  in reduced form; I and X are the hypothetical intermediates participating in phosphorylative exchanges (indicated earlier). Detailed discussion of this hypothesis has been given by Bronk (1973).

The chemiosmotic hypothesis suggests that the excess of  $H^+$  on the outer side of the mitochondrial membrane may initiate the formation of ATP by means of an ATPase driven reaction in reverse direction. It has been shown that mitochondrial ATPase is so orienta-

ted that  $H^+$  accumulates on the outer surface by the following reaction:



The  $H^+$  concentration at the outer surface is further increased, if the respiratory chain oxidation is also producing  $H^+$  at the outer surface. At the same time the  $H^+$  concentration at the inner surface of the mitochondrial membrane declines due to accumulation of  $CH^-$ . Thus the ratio of  $H^+$  at the outer surface to  $H^+$  at the inner surface is quite high at the equilibrium, which drives the reaction catalyzed by ATPase towards ATP synthesis. A detailed treatment to this hypothesis has been given by James (1971) and Bronk (1973).

## UTILIZATION AND METABOLISM OF NITROGEN SOURCES

The role of nitrogen in fungal physiology has received considerable attention of the mycologists during the last three decades. The account has been well illustrated by Foster (1949), Hawker (1950), Lilly and Barnett (1951) and Cochrane (1958). Due to vague and contradictory biochemical data it is difficult to computerize the findings under a single orbit. Contradictions in the conclusions arise mainly because every nitrogen source used in the culture medium undergoes complex transformations, which vary with the nature of the organisms and the experimental set up. Like carbon sources, nitrogen is also used both for functional as well as structural purposes by fungi. The form of nitrogen has a profound effect on metabolism of micro-organisms. Literature is full with conflicting claims regarding the comparative superiority of a particular form or source of nitrogen over the other. Specificity for the choice of nitrogen is more pronounced in some and less in other organisms. Occasional attempts have also been made to classify the fungi on the basis of their nitrogen requirements. The classification which needs attention is that of Robbins (1937) who grouped fungi in four categories on basis of their capacity to utilize nitrate, ammonium, organic and elemental nitrogen. This classification is tabulated, as follows:

<i>Nature of Organism</i>	<i>Nitrogen Source</i>			
	<i>Nitrate</i>	<i>Ammonium salts</i>	<i>Organic nitrogen</i>	<i>Gaseous nitrogen</i>
I. Nitrogen fixing organisms	+	+	+	+
II. Nitrate-ammonium utilizing organisms	+	+	+	-

III. Ammonium utilizing organisms	—	+	+	—
IV. Organic nitrogen utilizing organisms	—	—	+	—

(+) indicates capacity to utilize the source.

(-) indicates incapacity to utilize the source.

Robbins suggested his classification about four decades ago and some questions have been raised regarding its applicability at the present moment. Cochrane (1958) has expressed his doubt if any fungus is really attributable to group I of Robbins classification. Similarly, the fungi capable of using nitrate, ammonium and organic nitrogen have been placed in group II, which obviously suggests that those which are capable of utilizing nitrate nitrogen can use ammonium nitrogen also. But, *Cryptococcus nigricans* (Rich and Stern, 1958) is an exception, because it uses nitrate but not ammonium nitrogen. Similarly, the fungi placed in group IV are capable of using only organic nitrogen. Many recent studies suggest that the ammonium nitrogen for many fungi becomes unutilizable because of rapid fall of pH of the medium. The source, however, becomes utilizable if the medium is supplemented with salts of certain organic acids, like fumaric acid or succinic acid. The demarcating line between group III and group IV is not so sharp as conceived by Robbins. The classification of Robbins seems to be losing its importance due to above reasons and also because of the differences that have been observed in the nutritional response of closely allied species and sometimes even among different isolates of the same species. More detailed and comprehensive studies on nitrogen-nutrition dealing with a large number of diverse fungal forms might be rewarding in suggesting an improved and more valid classification of fungi on the basis of their nitrogen requirements.

Studies dealing with nitrogen metabolism of fungi are full of gaps and the exact mechanism of nitrogen assimilation into amino-acids and proteins and synthesis of specific peptides as well as the metabolism of purines and pyrimidines leave many questions unanswered. Details of the metabolism will be discussed later.

### UTILIZATION OF NITROGEN SOURCES

**Nitrate.** In general, nitrates have been reported to be excellent sources for imperfect fungi and Ascomycetes (Lilly and Barnett, 1951; HacsKaylo *et al.*, 1954; Thind and Randhawa, 1957; Suryanara-

yanan, 1958; Misra and Mahmood, 1960; Agarwal and Ganguli, 1960; Bilgrami, 1964; Tandon, 1967; Narasimhan, 1969; Subramanian and Tyagi, 1968). Higher Basidiomycetes are generally incapable of utilizing it, while some members of this group showed feeble response when long incubation periods of seventy days or more were used. Among the Phycomycetes, species of *Pythium* (Saksena *et al.*, 1952; Grover and Sidhu, 1965) show a favourable response towards the nitrate nitrogen. Saprolegniales (Bhargava, 1945; Reischer, 1951), Blastocladales (Cantino, 1955), as well as two marine Phycomycetes, *i.e.* *Thraustochytrium motivum* and *T. multirudimentale* (Goldstein, 1963) do not grow on nitrate nitrogen. Incapacity to use nitrate nitrogen is usually considered to be absolute in some of the fungi. However, there exists a possibility that at least some of such forms might be capable of metabolising nitrate nitrogen in later stages if initial growth is attained at the expense of some readily available nitrogen source. Cochrane (1950) observed that spores of *Streptomyces griseus* were incapable to grow on nitrate medium but a pregrown mycelium could use nitrate readily. It is obvious that in such studies the nitrate-reducing enzymes are induced in the pre-formed mycelium, after it is inoculated in a nitrate containing medium. There are also some reports (Raper *et al.*, 1945; Pontecorvo, 1953; Sakaguchi and Ishitani, 1952), which suggest that capacity to use nitrate is sometimes lost by mutation. Efficiency of different forms of nitrate varies for fungi. Tandon (1967) reported that sodium nitrate, calcium nitrate and magnesium nitrate were generally inferior to potassium nitrate for Fungi Imperfecti. Difference in the value of various types of nitrates is obviously due to different cations involved in these compounds. There are several reports (Lindeberg, 1944; Norkrans, 1950; Fergus, 1952; Bilgrami, 1964; Tandon, 1967) to suggest that within a genus individual species differ for their nitrate utilization. The capacity to use nitrate by fungi actually depends upon their nitrate reductase activity.

Ammonium nitrate has also found extensive application in fungal nutrition as nitrogen source. This substance is reported to be inferior to potassium nitrate for a large number of imperfect fungi (Mix, 1933; Durairaj, 1956; Suryanarayanan, 1958; Bilgrami, 1964, and Tandon, 1967). A pronounced fall in pH of ammonium-nitrate medium during the growth of fungi is quite common (Isaac, 1949; Haskins and Weston, 1950; Pelletier and Keitt, 1954; Srivastava, 1955; Narsimhan, 1969). This is an indirect evidence about the preferential utilization of ammonium ion. Analytical studies with

*Scopulariopsis brevicaulis* (Morton and MacMillan, 1954) and *Alternaria tenuis* (Srivastava, 1955) have shown that nitrate utilization did not start till virtually all ammonium had been exhausted from the medium. Somewhat different data have been collected for *Helminthosporium gramineae* (Converse, 1953), where slight assimilation of nitrate alongwith utilization of ammonia has been reported.

Other ammonium salts like ammonium sulphate, ammonium carbonate and ammonium chloride are generally inferior to ammonium nitrate in their nutritive value. Utilization of ammonium salts by fungi invariably results in rapid drop of pH of the culture solution. This has been recorded during the growth of several fungi including *Chytridium* sp. (Crasemann, 1954), *Pythium* sp. (Saksena *et al.*, 1953), *Choanephora cucurbitarum* (Barnett and Lilly, 1956), *Penicillium* sp. (Curtis *et al.*, 1951), *Myrothecium verrucaria* (Darby and Mandels, 1954); *Alternaria tenuis* (Srivastava, 1955), and *Sclerotium rolfsii* (Narsimhan, 1963). Ammonium utilization by *Scopulariopsis brevicaulis* (Morton and MacMillan, 1954) unlike nitrate absorption has no definite pH optimum. Ammonia enters and leaves the cell by passive diffusion as undissociated  $\text{NH}_3$  molecule. Rapid fall in pH due to assimilation of ammonical nitrogen results in cessation of growth of many fungi (Cochrane, 1958; Nicholas 1963). Addition of certain organic acids to media containing ammonium salts generally results in resumption of growth. Significant improvement in growth of *Phyllosticta* species (Bilgrami, 1964) was recorded when ammonium sulphate medium was supplemented with sodium succinate or sodium fumarate. There is appreciable controversy in the literature regarding the mode of action of some of these organic acids or their salts. Leonian and Lilly (1943) as well as Brian *et al.* (1947) were of the view that fungi studied by them possessed specific requirement for particular types of organic acid; Morton and MacMillan (1954), however, considered that their main function was due to buffering, which prevented rapid fall in pH. Srivastava (1955) observed that sodium or potassium salts of succinic or fumaric acid permitted complete assimilation of ammonium nitrogen by *Alternaria tenuis*, though the final dryweight was influenced by the nature of the organic acid supplied. Subramanian (1961), Sethunathan (1964) and Tyagi (1967) have also discussed the role of organic acids in facilitating the fungal growth on ammonium salts. Some possibilities about toxicity of ammonia have also been suggested and organic acids are supposed to antidote the toxic actions. This view seems to have some validity only when concentration of ammonia is very high. The toxicity of high ammonium concentration is influenced by several



factors such as the nature of the carbon source, presence of amino acids and phosphates etc. conflicting and mostly semiquantitative data on this aspect do not permit to reach any decisive conclusion. Use of mixed nitrogen sources has also been suggested as a remedy for better utilizability of the ammonium compounds. Work on *Sclerotium rolfsii* (Narsimhan, 1963) indicates that this fungus can grow and utilize ammonical nitrogen even at low pH levels. This is perhaps the main reason for wide geographical distribution of *Sclerotium rolfsii*, which is a well known tolerant of low pH (Subramaniam, 1967). Better growth of some fungi (Hawker, 1950; Thind and Randhawa, 1957; Agarwal and Ganguli, 1960) on ammonium phosphate than on ammonium sulphate or ammonium chloride is attributed to comparatively slower change of media (containing ammonium phosphate to inhibiting hydrogen ion concentration. Ammonium assimilation generally leads to formation of glutamic acid in fungi, while other amino acids are subsequently formed from glutamate by transamination. The details have been discussed later.

**Nitrite.** Nitrite does not support the growth of many fungi which is attributed to know toxicity of this substance. Some fungi including *Phymatotrichum omnivorum* (Blank and Talley, 1941), *Scopulariopsis brevicaulis* (Morton and MacMillan, 1954), *Fusarium coeruleum* (Agarwal, 1955), *Botryodiplodia theobromae* and *Diplodia typhina* (Tandon, 1967) are able to use nitrite as the sole source of nitrogen. Detailed investigations with *Fusarium coeruleum* (Tandon and Agarwal, 1953) and *Botryodiplodia theobromae* (Tandon and Srivastava, 1963) indicated that hyphal output was maximum at alkaline pH. *Drechslera sorokiniana* (Tyagi, 1967, b) attained good growth on nitrite nitrogen if initial pH was adjusted to 7.5. The growth continued an ascending trend even when its concentration was raised to 20 mg. Growth of *Curvularia verruciformis* and *Fusarium moniliforme* (Sahni, 1967) substantially increased on nitrite solution, when pH was adjusted to 6.5 or more. More favourable growth on alkaline nitrite media suggests that it is the free unionized acid rather than nitrite ion which is toxic to fungi. Accumulation of pyruvic acid due to nitrite toxicity has been reported in *Fusarium lini* (Nord and Mull, 1945). Morphological changes in some fungi (Steinberg and Thom, 1942) on nitrite containing media is also recorded. Since nitrite is an intermediate product in the utilization of nitrate, ordinarily it is expected that organisms which utilize nitrates well should be able to use nitrite equally well if not better. *Sclerotium rolfsii* (Sahni, 1967) grows well in nitrite medium but is incapable of growing even at alkaline levels of nitrite medium. This is possibly due to

lack of specific carrier enzyme systems (Guirad, 1958; Cirrillo, 1961) which are required to transport the nitrite within the cell to a suitable site where it could be reduced and utilized. *Scopulariopsis brevicaulis* (Mortan and MacMillan, 1954) grows well on potassium nitrite and its growth-response is quite distinct from that on ammonium nitrite with no corresponding drop in pH on the former compound. This is possibly an indication that uptake of ammonia and nitrite by the above fungus was simultaneous as well as at comparable rates. A similar relation between nitrite and ammonia was recorded when ammonia was added to a fast growing culture of *S. brevicaulis* in a nitrite medium. It could be concluded on basis of available data that in majority of the fungi the utilization of ammonium and nitrite compounds is influenced by the pH of the medium.

*Amino acid and amides.* A large number of amino acids are recorded as good nutritional sources for fungi. These substances are generally assimilated as such and they exist in fungal mycelium both in free as well as in bound state (Venkataram, 1956; Tandon and Bilgrami, 1960; Agnihotri and Mehrotra, 1961, Chandra and Tandon, 1962; Narsimhan, 1969). Nitrogen and carbon contents of the medium cause pronounced qualitative and quantitative variations in the amino acid contents of the mycelium (Tandon and Bilgrami, 1960; Narsimhan, 1969). Closely allied fungal species are known to differ in the amino acid composition (Crossan and Lynch, 1958; Bilgrami, 1962). Few efforts have been made to classify fungi on basis of the amino acid contents of their mycelium. Amino acid contents in species of *Colletotrichum* (Crossan and Lynch, 1958) also reflect their degree of virulence to certain extent. Their rate of assimilation from the supporting medium varies with the nature of the micro-organism, other ingredients of the medium and experimental set-up. Leaching of amino acids from hyphae is quite frequent specially when autolysis sets in (Pyle, 1954; Pfenning, 1956). This is generally the reason for the culture solutions to get enriched with free amino acids during the late incubation period.

Amino acids as the sole source of nitrogen have been used for numerous fungi. Most fungi utilize amino acids directly and are incorporated into proteins as such, without undergoing enzymatic degradation. Possibly on this account free amino acids are readily detectable in fungi. Except for certain broad generalization, their

nutritive capacity differs with the organisms. This is partly because some fungi meet a part of their carbon requirement also from the amino acids. Various factors like permeability, their contamination with vitamins, their enzymatic capacity etc., are also attributed as the possible reasons for such varied behaviour. Data on this aspect are substantially conflicting (Pyle, 1954; Tandon and Grewal, 1956; Skoropad and Arny, 1957; Thind and Randhawa, 1957; Kurtz and Fergus, 1964; Grover, 1964; Lopez and Fergus, 1965; Prasad, 1965; Hasija, 1966; Mathur *et al.*, 1967; Ram Dayal, 1968; Srivastava and Tandon, 1969). In general, short chain amino acids like glycine, DL-alanine, L-glutamic acid and DL-aspartic acid are listed as good sources for a large number of imperfect fungi (Tandon, 1967), species of *Aspergillus* (Mehrotra and Agnihotri, 1963); and Mucorales (Sarbhoj, 1963). Common amino acids present in a host generally supported good growth of pathogenic species of *Phyllosticta* (Bilgrami, 1962). Aspartic acid was found to be inferior to its amide asparagine (which is an important reserve constituent of green plants) for a large number of leaf spot fungi (Bilgrami, 1963). Similar superiority for amide nitrogen is known for *Pyricularia oryzae* (Otani, 1952), *Tricholoma imbricatum* (Norkrans, 1953), *Aspergillus flavus* (Grover, 1963), *Botryodiplodia theobromae* (Prasad, 1965), *Colletotrichum* sp. (Hasija, 1966), *Cercospora jasminicola* (Dayal and Ram, 1968). Mycelial mats of *Drechslera sorokiniana* had maximum accumulation of nitrogen when cultivated on asparagine (Subramanian and Tyagi, 1968). Wolf (1951), Beckman *et al.* (1953), Jennison *et al.* (1955) and Bilgrami (1962) reported that amino acid and amide forms were almost of equal value. Meister (1955) observed that in some systems asparagine was more active than asparatate as amino group donor in transamination. Similarly glutamine is also reported to be superior to glutamic acid for several fungi (Tandon, 1967). Whether this is due to the extra nitrogen present in the amides, or their greater buffering effect on the medium is not yet clear. Possibility of pH effect of the medium in the utilization of amide and amino nitrogen also exists.

L-Proline is also listed as a good source in fungal nutrition (Wolf, 1953; Davis, 1955; Leaphart, 1956; Thind and Randhawa, 1957). Traces of glutamic acid were spotted from the second day in the medium containing proline during the growth of *Phyllosticta* spp. (Bilgrami, 1962). Rapid transformation of proline to glutamic acid is possibly the reason for better efficiency of this amino acid. Two

sulphur containing amino acids, cysteine and methionine are generally poor sources. Increased growth on these compounds is often related to sulphur requirement of the fungi. Nutritive value of some common amino acids for fungal species are detailed in Table 10.1.

TABLE 10.1

Amino Acid	Good Source for	Poor Source for
Glycine	<i>Aspergillus rugulosus</i> , <i>A. violaceus</i> (Mehrotra and Agnihotri, 1963) Five isolates of <i>Piricularia oryzae</i> (Mathur <i>et al.</i> , 1967) Five species of <i>Zygorhynchus</i> (Sarbhoy, 1964).	Two species of <i>Zygorhynchus</i> (Sarbhoy, 1964).
DL-Serine	Five species of <i>Aspergillus</i> (Mehrotra and Agnihotri, 1963) <i>Colletotrichum inamdarii</i> (Hasija, 1966) <i>Pestalotia algeriensis</i> , <i>P. vismae</i> (Mitra and Tandon, 1970).	<i>Colletotrichum gloeosporioides</i> (Prasad, 1965) <i>Pestalotia bicolor</i> (Mitra and Tandon, 1970).
DL-Valine	<i>Aspergillus rugulosus</i> (Mehrotra and Agnihotri, 1963) <i>Curvularia pallescens</i> (Hasija, 1970 a) <i>Alternaria tenuis</i> (Singh & Tandon, 1967) <i>Colletotrichum gloeosporioides</i> (Tandon and Verma, 1962).	
DL-Leucine	<i>Aspergillus violaceus</i> (Mehrotra & Agnihotri, 1963) <i>Colletotrichum gloeosporioides</i> (Tandon & Chaturvedi, 1962) <i>Chytrium hyalinus</i> (Hasija and Miller, 1971).	<i>Aspergillus quadrilineatus</i> (Mehrotra & Agnihotri, 1963) <i>Colletotrichum capsici</i> (Tandon & Chaturvedi, 1962). <i>Phyllosticta artocarpina</i> (Tandon & Bilgrami, 1957).
DL-Isoleucine	<i>Aspergillus rugulosus</i> (Mehrotra and Agnihotri, 1963).	<i>Aspergillus nidulans</i> (Mehrotra and Agnihotri, 1963).
DL-Alanine	<i>Aspergillus nidulans</i> , <i>A. rugulosus</i> , <i>A. variegatus</i> (Mehrotra & Agnihotri, 1963) <i>Myrothecium roridum</i> (Chauhan & Suryanarayanan, 1970) <i>Curvularia pallescens</i> (Hasija, 1970).	<i>Aspergillus quadrilineatus</i> (Mehrotra and Agnihotri, 1963). Five isolates of <i>Piricularia oryzae</i> (Mathur <i>et al.</i> 1967).

Amino Acid	Good Source for	Poor Source for
L-Glutamic acid	<i>Pyricularia oryzae</i> (Manibhushan Rao, 1971) <i>Alternaria tenuis</i> (Singh and Tandon, 1967) Three species of <i>Pestalotia</i> (Mitra and Tandon, 1970) Five species of <i>Aspergillus</i> (Mehrotra & Agnihotri, 1963).	<i>Cercosporina ricinella</i> (Tandon & Chandra, 1962) <i>Curvularia penniseti</i> (Tandon and Chandra, 1961).
✓ DL-Aspartic acid	<i>Curvularia pallescens</i> (Hasija, 1970 a) <i>Alternaria tenuis</i> (Hasija, 1970) Three species of <i>Zygorhynchus</i> (Sarbhoy, 1964).	<i>Aspergillus flavus</i> (Grover, 1964) <i>Alternaria citri</i> (Hasija, 1970) <i>Curvularia penniseti</i> (Agarwal, 1958). Two species of <i>Zygorhynchus</i> (Sarbhoy, 1964).
✓ L-Asparagine	<i>Helminthosporium rostratum</i> (Agarwal & Shinkhede, 1959) <i>Chytromyces hyalinus</i> (Hasija and Miller, 1971) Three species of <i>Phyllosticta</i> (Bilgrami, 1957) <i>Colletotrichum gloeosporioides</i> (Tandon & Verma, 1962) <i>Helminthosporium oryzae</i> (Misra and Mukherjee, 1962).	<i>Gloeosporium psidii</i> , G. citricolum (Agarwala, 1955).
✓ L-Glutamine	<i>Tricholoma gambosum</i> (Norkrans, 1953) Species of <i>Phyllosticta</i> (Bilgrami, 1963).	
/ L-Histidine	<i>Venturia inaequalis</i> (Pelletier and Keitt, 1954).	<i>Myrothecium roridum</i> (Chauhan and Suryanarayanan, 1970) <i>Phyllosticta artocarpina</i> (Tandon & Bilgrami, 1957) <i>Alternaria tenuis</i> (Grewal, 1955).
L-Tyrosine	<i>Myrothecium roridum</i> (Chauhan and Suryanarayanan, 1970) <i>Helminthosporium carbonum</i> (Ram Dayal & Joshi, 1969).	

<i>Amino Acid</i>	<i>Good Source for</i>	<i>Poor Source for</i>
L-Arginine	<i>Cercosporina ricinella</i> (Tandon and Chandra, 1962 a) <i>Colletotrichum gloeosporioides</i> (Tandon & Chandra, 1962 b).	<i>Phycomyces blakesleeana</i> (Lilly and Barnett, 1951).
DL-Tryptophan	<i>Myrothecium roridum</i> (Chauhan & Suryanarayanan, 1970) <i>Aspergillus flavus</i> (Grover, 1964).	<i>Venturia inaequalis</i> (Peltier and Keitt, 1954).
L-Proline	Three species of <i>Pestalotia</i> (Mittra and Tandon, 1970).	
DL-Methionine		<i>Alternaria tenuis</i> (Grewal, 1955).
L-Cysteine		<i>Myrothecium roridum</i> Chauhan and Suryanarayanan, 1970).

It is evident from the above records that amino acids substantially differ in their nutritive value for fungi. Such variations are known to occur among different strains of the same species. This point has been thoroughly elaborated by Tandon (1967) for different species of *Colletotrichum*, *Gloeosporium*, *Pestalotia* and *Phyllosticta*.

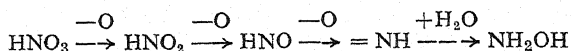
Mixture of amino acids is generally reported to be superior to any individual amino acid source. In *Botryodiplodia theobromae*, (Srivastava and Tandon, 1969), *Colletotrichum gloeosporioides* (Tandon and Chaturvedi, 1962) and species of *Phyllosticta* (Bilgrami 1962) these conclusions are based on regular chromatographic analysis of the supporting medium. Grover (1964) recorded substantial increase in the hyphal output of *Aspergillus flavus* on a mixture of the amino acids. For some fungi (Lilly and Barnett, 1951) mixture may be inferior to an individual source of amino acid.

Some times mutation also induces requirement for specific amino acid among fungi, but it is uncommon in nature, and even when it occurs it is more often partial rather than absolute requirements. Utilization of D-isomers of amino acids by fungi leads to activity of D-amino acid oxidase resulting in release of ammonia which subsequently gets incorporated into L-amino acids. Racemases, which interconvert D- and L- forms are known in bacteria but not so far located in fungi.

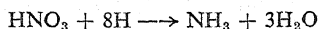
## NITROGEN METABOLISM

### Metabolism of Nitrate

Nitrate reduction in fungi is to a great extent similar to that reported in bacteria and chlorophyllous plants. This is carried out by a group of enzymes in various steps, ultimately leading to formation of ammonia. Nitrite, nitric oxide, hyponitrite and hydroxylamine are the principal intermediate substances, which are produced during the course. A scheme for nitrate reduction based only on chemical considerations was suggested as early as in 1896 by Bach:



Except the first stage, none of the stages suggested by Bach had any biological evidences at that time. Reduction of nitrate to ammonia requires a total of eight hydrogen atoms or electrons:



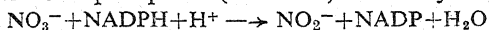
It is now generally known that in biological oxidation and reduction, electrons are removed or added in pairs. This amply suggests the reduction of nitrate to ammonia to be a four step process, as shown below:



Initially, perfect evidences for enzymatic catalysis were available only for the first and the last steps, but recent researches have more or less established the involvement of enzymes at all the stages. The task of nitrate reduction is accomplished by a group of inducible enzymes which are generally metallo-flavoproteinaceous in nature, where flavin adenine dinucleotide (FAD) is the prosthetic group. Role of the various enzymes operative during different stages of nitrate-reduction by fungi is discussed as under.

**Nitrate reductase.** Nitrate reduction in fungi is carried out by nitrate reductase enzyme. This enzyme was formerly considered to be only assimilatory in nature but subsequently their dissimilatory nature was also established (Walker and Nicholas, 1961) and in some fungi they are functional even under semianaerobic condition when oxygen supply is limiting or scanty. Most valuable information on this aspect has been obtained from *Neurospora crassa* (Evans and Nason, 1952; Nason and Evans, 1953). The enzyme is operative only when *N. crassa* is cultivated on nitrate or nitrite media, which obviously reflects the inducible nature of nitrate reductase. It was obtained in a partially

purified state by Nason and Evans (1953), who noted that the enzyme was capable of reducing nitrate to nitrite, with reduced nicotinamide adenine-dinucleotide phosphate (NADPH) as the hydrogen donor.



It was Steinberg who for the first time in 1937 showed that *Aspergillus niger* required molybdenum, when grown with nitrate as the sole nitrogen source. The findings were subsequently confirmed by Nicholas and Fielding (1951). It has been established that in the above reaction flavin adenine-dinucleotide (FAD) is the prosthetic group, sulfhydryl group is essential to activity, while molybdenum is an essential factor in electron transport. Various workers (Nicholas *et al.*, 1954; Nicholas and Nason, 1954 a, b; Nicholas and Stevens, 1955; Nicholas, 1959 a, b) with a series of experiments established that molybdenum was a functional constituent of nitrate reductase and this metal links the flavin component to nitrate. Similar enzyme is recorded in the yeast, *Hansenula anomala* (Silver, 1957) and *Escherichia coli* (Nicholas and Nason, 1955 b). Nitrate reduction in *Neurospora* requires inorganic phosphate replaceable by arsenate, selenate, tellurate or tungstate but not by silicate or adenosine triphosphate (Nicholas and Scawin, 1956; Kinsky and McElroy, 1958). Possibly molybdenum occurs in enzyme system as phosphomolybdate. It is considered that two molybdenum atoms are required to cause the two-electron transfer to nitrate:

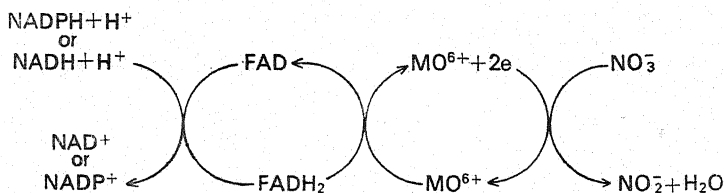


Fig. 10.1

Further evidence regarding the involvement of molybdenum in electron-transfer has been obtained with electron-paramagnetic resonance (E P R) studies. Kinsky and McElroy (1958) reported that in *N. crassa* the nitrate reductase activity was intimately linked with cytochrome system, particularly C-cytochrome. Subsequent investigation by Kinsky (1961) showed that NADPH, cytochrome-C-reductase and nitrate reductase displayed parallel induction and acti-



vity. Mutants lacking nitrate reductase activities were studied both in *Neurospora* and *Aspergillus* (Silver and McElroy, 1954; Sorger 1963), and it was demonstrated that FAD/molybdenum protein complex and the NADP/FAD/Cytochrome C were separately determined by non-allelomorphic genes nit-1 and nit-2 in *Neurospora*. It appears that the two reactions are functionally different.

In contrast to bacterial systems the dissimilatory nitrate reductase enzymes of fungi require iron as well as molybdenum for its activity (Fewson and Nicholas, 1961 b). Reduced methyl—or benzyl-viologen is the most effective hydrogen donor in this system, which does not require flavin. NADPH, which is an effective donor for the assimilatory enzyme does not function for the purified dissimilatory system. These two nitrate reductase systems (assimilatory and dissimilatory) differ only in the penultimate electron transfer sequence to the terminal nitrate reductase which is a molybdenum containing protein.

### Dissimilation

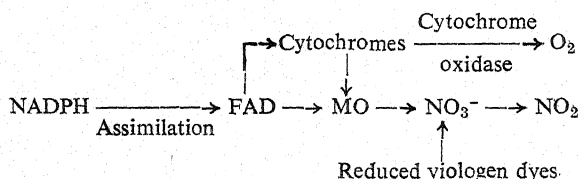
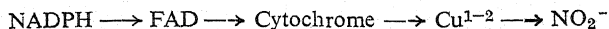


Fig. 10.2. Alternative pathways of electron transfer to nitrate and oxygen in fungi.

**Nitrite reductase.** Nitrite formation represents a transitional phase during nitrate reduction. Nason *et al.* (1954) succeeded in isolating a nitrite reductase from *Neurospora*, which was capable of reducing nitrite to ammonia, when NADH was the hydrogen donor. Like the nitrate reductases they are also metallo-flavoproteins with FAD as the prosthetic group. NADPH was, however ineffective as a donor. The enzyme was stimulated by FAD and was inhibited by metal-binding agents like cyanide and 8-hydroxyquinoline etc. Subsequently Nicholas *et al.* (1960) separated FAD, copper and iron from a highly purified fraction of this enzyme. They suggested that copper was needed for the ultimate step, coupling the electron transfer sequence to nitrite. During this reaction reduction of nitrite is accomplished

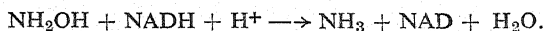
non-enzymatically by univalent copper while bivalent copper is subsequently reduced enzymatically by the penultimate donor system.



Nitrite reductase from *Neurospora* shows a close similarity in its properties to the nitrite reductase from denitrifying bacteria e.g. *Pseudomonas stutzeri* (Chung and Najjar, 1956 a). *Pseudomonas aeruginosa* (Walker and Nicholas, 1961 C). Both fungal as well as bacterial nitrite reductases have cytochrome oxidase activity. Nitric oxide is produced as a result of nitrite reductase activity of *Neurospora crassa*. Under an aerobic conditions nitric oxide is taken up by both bacterial as well as fungal organisms. Production of nitric oxide as an intermediate is now well established during bacterial denitrification. However, during nitrate assimilation by fungi, the production of nitric oxide enroute to ammonia is yet to be substantiated with perfect evidences.

*Hyponitrite reductase.* Medina and Nicholas (1957 b) isolated another enzyme i.e. hyponitrite reductase from *Neurospora*. Nicholas (1957 b) regard this enzyme to be distinct from nitrite reductase because zinc deficiency reduces the activity of hyponitrite reductase more sharply than nitrite reductase. The existence of hyponitrite reductase in other living systems is not yet substantiated though its existence would complete a logical set of enzymes reducing nitrate to ammonia.

*Hydroxylamine reductase.* *Neurospora* has been a source for hydroxylamine reductase (Zucker and Nason, 1955). This enzyme is also a metallo-flavoprotein with FAD as the active group. It reduces hydroxylamine according to the following equation.



Activity of this enzyme is reduced in a manganese or magnesium deficient medium (Medina and Nicholas, 1957 a). Medina and Nicholas (1957 b) determined the effects of metal deficiencies on nitrite, hyponitrite, and hydroxylamine reductase in *Neurospora*, on the basis of ammonia production. Observations made by them are recorded in Table 10.2.

It is clear from the records that metal deficiency affecting the hydroxylamine or hyponitrite systems also inhibited nitrite reductase activity. Thus a deficiency of manganese that reduced hydroxylamine reductase also depressed nitrite and hyponitrite reductases. The

TABLE 10.2

EFFECTS OF METAL DEFICIENCIES ON NITRITE, HYPONITRITE,  
AND HYDROXYLAMINE REDUCTASES IN *NEUROSPORA*  
*CRASSA* (BASED ON AMMONIA PRODUCTION)<sup>a, b</sup>

Reductase enzymes	Omit Fe	Omit Cu	Omit Mn	Omit Mo	Omit Zn
Nitrite	22	36	53	100	68
Hyponitrite	51	53	60	100	100
Hydroxylamine	100	100	57	100	95
Weight of mycelium	32	43	41	40	59

<sup>a</sup>—after Medina and Nicholas (1957 b).

<sup>b</sup>—Values as % of these in normal mycelium.

observations further showed that although Mn and Mg were necessary for the formation of enzyme, none of these metals accumulated in purified fraction of the enzyme nor they were able to stimulate the enzymic activity. It is quite likely that these two metals are required for formation of the enzyme rather than for its action.

Hydroxylamine finally gets converted to ammonia, which has been found to accumulate in a large number of fungal and bacterial cultures. Ammonia is regarded as the "key intermediate" in the final conversion of inorganic to organic nitrogen. Following pathway of nitrate utilization by *Candida utilis*, has been suggested.

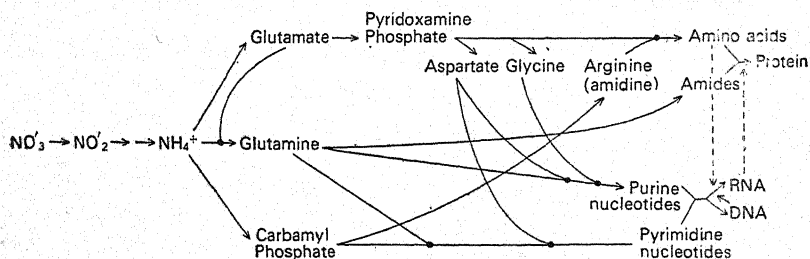
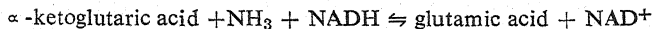


Fig. 10.3.

### Metabolism of Amino Acids

Ammonia which is the key intermediate in nitrate metabolism combines with carbon compounds to form organic nitrogenous substan-

ces. The details of the mechanism by which this key process proceeds is not known in most of the fungi. Studies on biosynthetic processes in germinating spores of some fungi lead to conclusion that skeletal material for amino acid synthesis is obtained as a result of catabolism of carbohydrate. Uredospores of *Uromyces phaseoli* and *Puccinia helianthi* furnished with 14 C-2 acetate, produced labelled glutamate, aspartate and alanine within a few seconds. It is generally accepted that reductive amination of  $\alpha$ -keto acids, followed by transamination is a major pathway of amino acid synthesis in living organisms. There is overwhelming evidence to show that glutamic acid is a central product in amino acid metabolism and many other amino acids are derived from it by known pathways. As early as 1925, Knoop and Osterbri showed that *in vitro* ammonia combines with oxalacetic acid and  $\alpha$ -ketoglutaric acid to form  $\alpha$ -iminoacids which is readily reduced to glutamic acid and aspartic acid. The occurrence of this process *in vivo* was confirmed by Adler *et al.* (1938) who detected in seven plant species a glutamic acid dehydrogenase dependent on diphosphopyridine nucleotides which catalysed the reversible formation of glutamic acid from  $\alpha$ -ketoglutaric acid according to the following equation:



Studies by Sims and Folkes (1963) dealing with *Candida utilis*, lead to the same conclusion. They supplied the above fungus with labelled ( $^{15}\text{NH}_4$ )<sub>2</sub> HPO<sub>4</sub> and noted that free glutamic acid and glutamine got rapidly labelled, and other amino acids like alanine, glycine, and aspartic acid are derived from them. Extending their studies further, on basis of kinetic analysis they established the formation and sequence of eighteen amino acids derived from glutamate (Table 10.3).

Fincham (1951) reported that *Neurospora crassa* contains a glutamic acid dehydrogenase, which is dependent on triphosphopyridine nucleotides. Later, Nicholas and Mabey (1960) reported that zinc was essential for formation of glutamic acid dehydrogenase. This enzyme is now known to be very widely distributed in nature.

Another possible synthetic route for amino acids is through reversal of their oxidative breakdown by general amino acid oxidases. Radhakrishnan and Meister (1958) showed the reversibility of reaction:

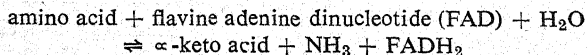


TABLE 10.3

THE ORIGINS OF  $\alpha$ -AMINO ACIDS IN *CANDIDA UTILIS*  
SUPPLIED WITH<sup>15</sup> N-AMMONIA IN EXPONENTIALLY  
GROWING CULTURES. (FROM SIMS AND FOLKES, 1963)

Primary (from $\text{NH}_3$ )	Secondary (from glutamic acid)	Tertiary from ornithine : apparent secondary from glutamic)	Quaternary (from citruline : apparent tertiary)
Glutamic acid Glutamine	Alanine Serine Threonine Aspartic acid Lysine Histidine Proline Glycine Valine Leucine Isoleucine Phenylalanine Tyrosine Methionine Ornithine	Citruline	Arginine

The general amino acid oxidases are supposed to oxidize a wide variety of amino acids. Bender and Krebs (1950) reported that *Neurospora crassa*, contains a general oxidase active on a wide range of L-amino acids. Flavin adenine dinucleotide is its prosthetic group. L-amino acid oxidases are also known in *Aspergillus niger* and species of *Penicillium* (Knight, 1948). It is considered that L-amino acid oxidase, though present in a number of fungi is not identical in every case.

The importance of dicarboxylic amino acids particularly glutamic acid occupying a key position in metabolic transformations of nitrogenous substances is because of their ready conversion from TCA cycle acids and particularly  $\alpha$ -keto acid. A host of amino acids are known to be derived from glutamic acid in a number of living systems inclu-



of glycine and glutamic acid serves as a suitable donor of the amino group. Glycine and serine are closely related in *Penicillium notatum* (Arnstein and Grant, 1954) and *Ermotheicum ashbyii* (Goodwin and Jones, 1956). Participation of these amino acids in a number of biochemical reactions concerned with their biosynthesis is well known in fungi. Possibility of other pathways for ornithine biosynthesis in *N. crassa* also exists (Fincham, 1953; Vogel, 1954). Participation of ornithine in ornithine cycle of the above fungus was established by Fincham and Boylen, (1957). The ornithine cycle which is operative in *N. crassa* may be schematized in Fig. 10.6.

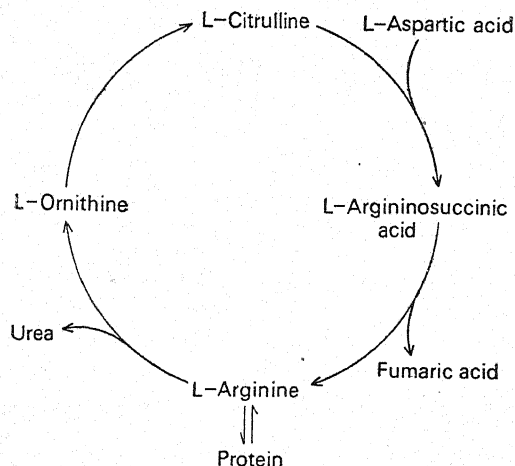


Fig. 10.6. Ornithine cycle.

Evidence of ornithine formation from glutamic acid has also been obtained in *Penicillium* (Bonner, 1946) and *Aspergillus* (Pontecorvo, 1950). According to Vogel (1955) ornithine synthesis in *Neurospora* and *Aspergillus* proceeds via glutamic semialdehyde but there is no evidence for acetylated intermediates as found in bacteria. Operation of ornithine cycle is now known in several fungi. Ornithine cycle primarily serves to supply arginine which is readily incorporated into protein.

Synthesis of sulphur containing amino acids, cysteine and methionine has been studied in four strains of *Neurospora crassa* by Horowitz (1947). By compiling the behaviour of all the four strains following sequence is suggested from cysteine to methionine.

Cysteine → Cystathionine → Homolysteine → methionine

It is also suggested that aspartic acid and homoserine are the precursors of homocysteine and methionine as in yeast and bacteria (Black and Wright, 1955). These workers presented the following scheme (Fig. 10.7) to depict the relationship of aspartic acid, threonine, cysteine and methionine:

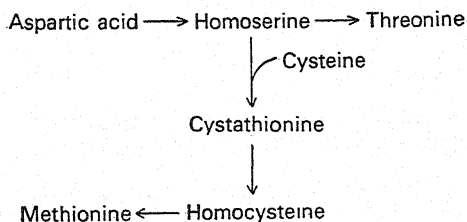


Fig. 10.7.

Watanabe and Shimura (1955) reported the existence of an enzyme in fungi which was capable of converting homoserine in threonine.

Yanofsky and Reissig (1953) reported that serine dehydrase from *N. crassa* caused non-oxidative deamination of L-serine and L-threonine which resulted in formation of pyruvate and  $\alpha$ -keto-butyrate respectively. D-amino acid oxidase from *Neurospora* is, however, incapable of attacking either D-serine or D-threonine.

Aspartase from *Penicillium notatum* (Tsuda, 1950) and *Neurospora crassa* (Shepherd, 1951) is known to convert aspartate to fumaric acid and ammonia non-oxidatively. The two dihydroxy acids *i.e.*  $\alpha,\beta$  dihydroxy- $\beta$  ethylbutyric acid and  $\alpha,\beta$  dihydroxy- $\beta$  methyl butyric acid, which arise from pyruvic acid and threonine are considered to be the precursors of isoleucine and valine, respectively (Adelberg, 1955). The keto analogue of leucine *i.e.*  $\alpha$ -ketoisocaproic acid is considered to be the precursor of leucine because it replaces this amino acid in a mutant of *Neurospora*.

Aromatic amino acids like tyrosine and phenyl alanine are derived from shikmic acid in *Neurospora* (Tatum *et al.*, 1954).

Some fungi like *Neurospora crassa*, *Aspergillus niger* and few species of *Penicillium* are known to attack tyrosine. The enzyme is referred as tyrosinase, which is a polyphenol oxidase and is supposed to operate more or less in the same manner as in a mammalian system. Tyrosine is oxidized via 2, 4-dihydroxyphenyl alanine through a red holochrome pigment to an insoluble black pigment, a melanin.



Isolation of tyramine from the cultures of *Aspergillus niger* (Uremura, 1939) suggests the breakdown of tyrosine by decarboxylation which is a common pathway in bacteria.

Yanofsky (1952) showed that in *Neurospora*, a phosphopyridoxal enzyme (tryptophan synthetase) catalyses the condensation of indole and serine to tryptophan. *Neurospora* is also known to synthesize tryptophan from nicotinic acid (Beadle *et al.*, 1947) and anthranilic acid (Tatum *et al.*, 1944). Tryptophan synthesis in *Claviceps purpurea* (Tyler and Schwarting, 1953) is probably effected in the same manner as in *Neurospora*.

Tryptophan break down by *Neurospora* leads to biogenesis of nicotinic acid with 3-hydroxykynurenine as an intermediate. Bonner and Yanofsky (1951) have reviewed this account and suggested the following scheme (Fig. 10.8) for synthesis of nicotinic acid.

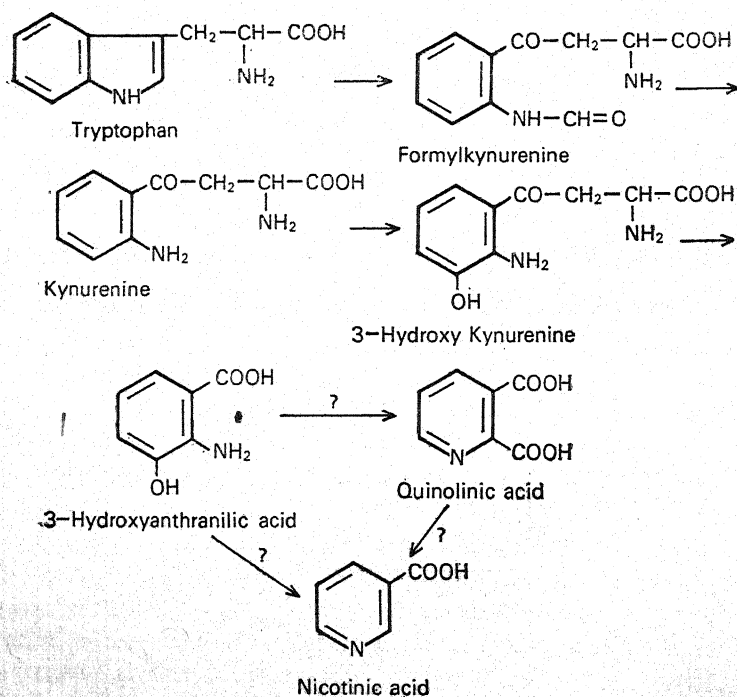


Fig. 10.8.

There is also overwhelming evidence to show that tryptophan is a precursor of indoleacetic acid which is synthesized by a large number of fungi.

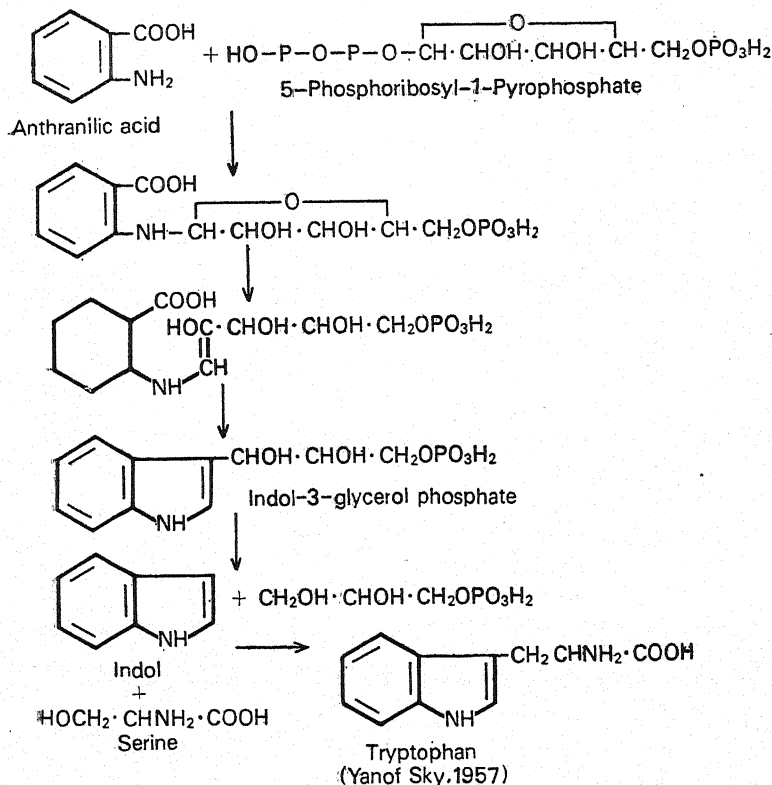


Fig. 10.9. Tryptophan Synthesis.

Studies with labelled metabolites indicate that formic acid, glucose and acetic acid are the efficient precursors of individual carbon atoms of histidine. These precursors have to undergo considerable transformations to produce the histidine molecule or its precursors containing the imidazole ring. The possible sequence of histidine synthesis by *Neurospora* is depicted (Fig. 10.10).

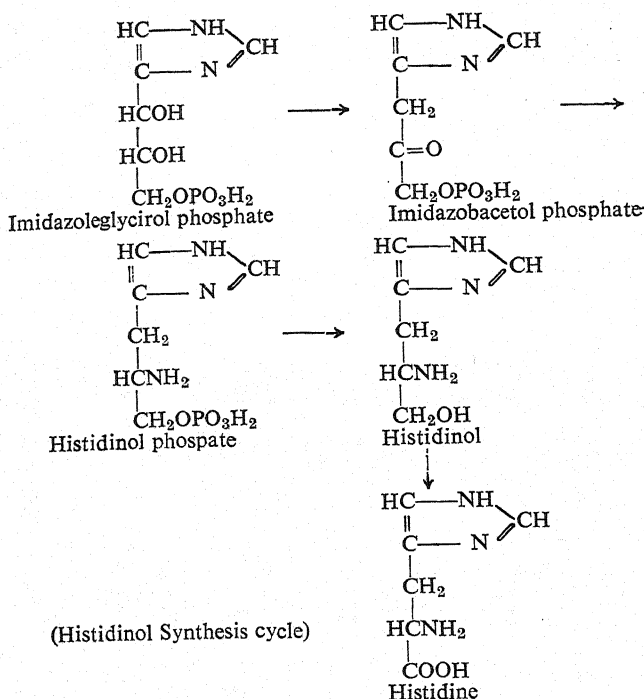


Fig. 10.10. Histidine synthesis cycle.

The exact sequence of histidine break down in fungi has not been investigated. In bacterial systems it is known to proceed via uronic and glutamic acids.

A review of literature on nitrogen metabolism of fungi makes it clear that the subject has been dealt mainly on basis of studies on *Neurospora crassa*. This is a fertile field which needs exploration with other fungi.

Glutamic dehydrogenase has been reported from several fungi including *Blastocladiella emersonii* (Mccurdy and Cantino, 1960), *Puccinia helianthi* (Smith, 1963). This enzyme has received special attention in *Neurospora*, *Penicillium* and *Ophiostoma*, where mutants deficient in glutamic dehydrogenase have been obtained.

Roberts *et al.* (1955), Cowie and Walton (1956) as well as Cowie (1962) furnished  $^{14}\text{C}$ -fructose to *Candida utilis* and obtained a group of amino acids from which other amino acids were later derived. Such results led to the concept of amino acid families,



substances actually depends on their efficiency to convert them into amino acids. Fischer (1902) and Hofmeister (1902) independently suggested that amino acids condensed to form peptides, which by further condensation formed the proteins and thus emerged a polypeptide hypothesis about protein composition. Casein (from cheese), albumen (egg white) and gelatin are some of the common proteins to be evaluated for their nutritional value to microbes under laboratory conditions. Commercial peptone, which is a mixture of peptides of varying chain length, has also been used extensively as a nitrogen source for fungi and bacteria. It is generally a useful source of nitrogen. The wide spread occurrence of enzymes hydrolysing peptides to amino acids indicates that in general the simple linear peptides are easily utilizable substrates for most of the fungi. Keratin utilization is generally restricted to dermatophytic fungi.

Enzyme affecting the cleavage of peptide bond has been termed as protease which has been distinguished into two types *i.e.* Endopeptidase and Exopeptidase.

(i) Endopeptidases act on peptide bonds either in simple peptides or in the interior of a protein chain.

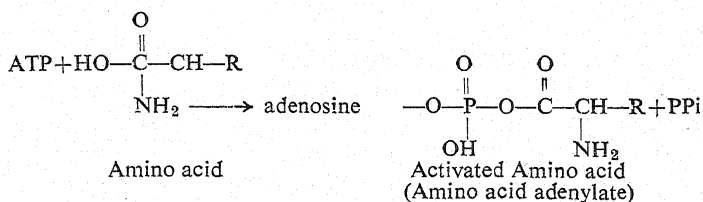
(ii) Exopeptidases act only on peptide bonds which are adjacent to a free  $\alpha$ -amino or a free carboxyl group and, therefore, restrict generally to hydrolysis of small peptides. Waksman and Starkey (1932) carried out extensive studies on utilization of proteins by fungi and bacteria. *Trichoderma koningi* and *Rhizopus* sp. are able to decompose casein, zein, gliadin and gelatin. Successful utilization of gelatin is also recorded for a large number of imperfect fungi (Maxwell, 1950; Dworschack *et al.*, 1952; Dingle and Solomons, 1952).

Some protein hydrolysing enzymes have been obtained from fungi. Enzymes even from closely allied species/strains are known to vary in their efficiency with regard to rate of attack on different types of proteins. Three different protein hydrolysing enzymes have been obtained from *Aspergillus oryzae* (Crewther and Lennox, 1953 a, b).

Protein-synthesis in the living organisms involves various steps which have been designated as (a) transcription stage, (b) activation stage, (c) adaptor stage, and (d) translation stage. During the transcription stage the genetic characters possessed by the DNA are copied by the RNA molecules. DNA is supposed to act as a template directing the synthesis of RNA. We know that the bases cytosine, adenine and guanine are common to both DNA as well as RNA, while the fourth base of DNA *i.e.* thymine closely resembles the base uracil

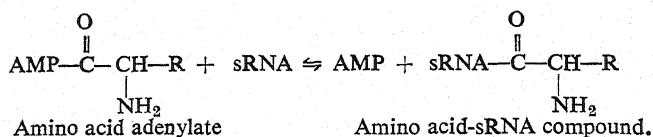
of RNA. This shows that nucleotide sequence in RNA and DNA will comprise of complementary strands except for the replacement of uracil by thymine unit. Thus the genetic information available in RNA is still coded as the nucleotide sequence. After its release from DNA the RNA moves into the cytoplasm, attaches to the ribosomal particles, and serves as the template for protein synthesis. This special type of RNA carries a copy or transcript of the genetic material from the DNA to the site (ribosomes) of protein synthesis and, therefore, it is designated as messenger RNA (*m* RNA).

During the second phase, there is activation and selection of amino acids for protein synthesis. This is accomplished by specific enzymes, which in presence of ATP catalyze the formation of an energy rich enzyme-bound amino acid adenylate (E-AA-AMP) and the release of pyrophosphate. During this process, a high-energy" acid-anhydride linkage is formed between the carboxyl group of the amino acid and the phosphate group of adenosine monophosphate, AMP.



In this manner, the carboxyl group of the amino acid gains increased reactivity at the expense of the cleavage of one of the phosphate anhydride bonds of ATP.

*Adaptation stage.* At this phase, the activated amino acid molecules attach themselves to RNA which result in the formation of special kind of RNA, generally referred to as soluble RNA (sRNA) or sometimes also called as transfer RNA. Berg *et al.* (1958) on basis of substantial evidence suggested attachment of amino acids to specific sRNA molecule. The point of attachment between sRNA and activated amino acid is considered to be at the 2nd or 3rd carbon of the ribose sugar of a terminal adenylic acid. sRNA molecules are very small nucleic acids which occur freely in the cytoplasm of the cell. There is atleast one kind of sRNA molecule for each of the twenty kinds of amino acids incorporated into proteins. Each sRNA molecule will react with only one kind of amino acid adenylate as shown on page 230.



*Translation stage.* This is the final stage in synthesis of a peptide chain, during which the nucleotide language of mRNA is translated into the amino acid language of the peptide. In this process a ribosomal particle combines with one end of the mRNA-chain, and in the mean time, activated amino acids bound to sRNA molecule also get attached to mRNA, leading to the formation of peptide bonds. The synthesis of polypeptides begins at the end of the chain, which has a free amino group, and proceeds linearly toward the end with the free carboxyl group. sRNA molecules are released from the mRNA for further use after the discharge of their amino acids.

### UTILIZATION AND METABOLISM OF NUCLEIC ACID

Like polysaccharides and proteins, the nucleic acids are also a class of biological polymer or macromolecules that occur in all the living cells. The monomeric components of nucleic acids are termed as nucleotides, which unlike the monosaccharides and amino acids may undergo further hydrolysis to yield three sub-components: (i) a nitrogen-containing cyclic base, a pentose sugar and a molecule of phosphoric acid. Depending upon the component sugar, the nucleic acid may be ribonucleic acid (RNA, containing ribose sugar), or deoxyribonucleic acid (DNA, containing D-2-deoxyribose sugar). The two types of nucleic acids also differ in their nitrogen base composition. As mentioned earlier three nitrogen bases, (*viz.* adenine, guanine and cytosine are common to both DNA and RNA, but the fourth nitrogen base is different in the two nucleic acids; RNA containing uracil, while DNA contains thymine. The nucleotides joined together by sugar phosphate linkage.

Functions of the nucleic acids mainly concern with storage, transmission and use of genetic informations, upon which the continuation of cell-structure depends. As early as in 1941, Caspersson pointed out that RNA contents of cells was closely correlated with their ability to synthesize protein. Bonnet and Gayet (1950) cited evidences that ribonucleic acid in the intracellular granules of micro-organisms was involved in protein synthesis.



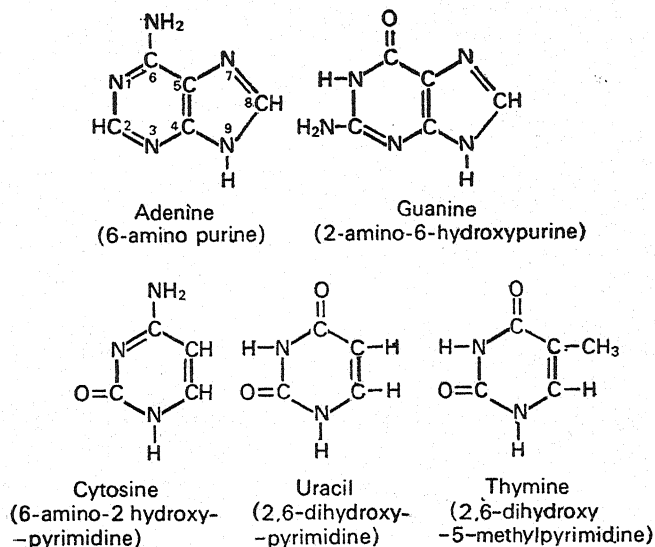


Fig. 10.12. Structure of nitrogen basis.

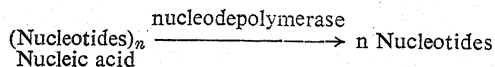
Nucleic acids and their derivatives have found limited application in fungal nutrition. Yet, it is generally recognised that their various components as well as the nucleic acids themselves are usually good sources of nitrogen for fungi (Wolf, 1953, 1955). A few variations in nutritional responses of some fungi have, also been recorded. Wolf (1953, 1955) recorded purines, pyrimidines and some methyl purines—caffeine and theophylline, as satisfactory, poor and unutilizable sources respectively for atleast two fungi, viz. *Neurospora sitophila* and *Fusarium oxysporum* f. *nicotianae*. Exogenously added adenine supports stimulated growth of a few basidiomycetous fungi (Fries, 1950 c, 1954; Jennison *et al.*, 1955). Likewise guanine and hypoxanthine (a hydroxypurine) exerts stimulatory influence on spore-germination and early growth of *Phycomyces blakesleeanus* (Robbins, 1940, 1941, 1943). Taha and Sharabash (1956) obtained good growth of various species of fungi with xanthine (a dihydroxypurine) as the sole source of nitrogen. Mutants deficient for purine or pyrimidine compounds have also been reported in diverse taxa of fungi (Loring and Pierce, 1944; Giles, 1946; Fries, 1947, 1948; Tomizawa, 1952).



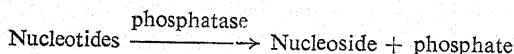
### Breakdown of Nucleic Acids and their Derivatives

The general pattern of nucleic acid degradation proceeds along the following sequence:

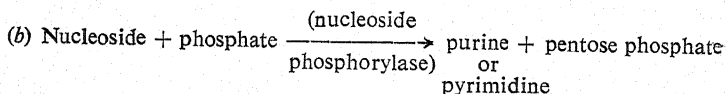
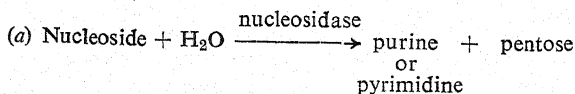
(i) Splitting-off of the polymer by appropriate nucleodepolymerase enzyme into monomeric nucleotides;



(ii) Dephosphorylation of the nucleotides by the action of phosphatase (the nucleotides may, however, be reutilized for nucleic acid synthesis);



(iii) Degradation of the nucleosides either by hydrolysis or by phosphorylation under appropriate enzymic activity;



The pentose sugar thus produced is further metabolized through pentose phosphate pathway, while the purines and pyrimidines are further degraded through different pathways. The purines (as known for animal systems) are degraded into uric acid allantoin or urea, while the pyrimidines are known to be completely broken down into carbon dioxide and ammonia.

Information on fungal degradation of nucleic acids is too scanty to provide a complete picture of this aspect. Yet, on the basis of a few reports on *Neurospora crassa* and some *Aspergilli* as well as similar information from bacteria, it is generally accepted that nucleic acid catabolism by fungi follows the same overall pattern involving the action of nucleodepolymerase and phosphatase enzymes. Enzymology of nucleic acid catabolism was initiated rather late with the discovery of the enzyme ribonuclease (Jones, 1920), which could hydrolyze RNA with gradual formation of free acid groups without producing any free phosphoric acid. Later, Laskowski, (1951) concluded that the ribonuclease enzyme was a nucleodepolymerase and it was widely distributed among fungi. Yet these enzymes have received only

scanty attention among fungi (Kunikana, 1954, 1955 a, b, c, 1957; Saruno, 1956; Sato and Egami, 1957; Heppel and Rabinowitz, 1958; Naoi-Tada *et al.*, 1959; Das Gupta and Verma 1961, 1962; Verma, 1963), although they (ribonucleases) are thought to play a significant role in cellular protein synthesis. Only a few fungi, including some species of *Alternaria* and *Aspergillus* have been screened for ribonuclease activity. Verma (1963) studied three strains of *Alternaria tenuis* and found that the ribonuclease enzyme was most active at pH 7.0 and temperature 40°C.  $\text{Cu}^{++}$  ions were found to stimulate the ribonuclease activity.

Deoxyribonucleic acids of fungi were first studied by Belozersky and his collaborators (Belozersky and Spirin, 1960; Uryson and Belozersky, 1960; Vanyushin *et al.* 1960). Recently a systematic account of DNA base composition of fungi belonging to diverse taxa has been provided by Storck and Alexopoulos (1970). However the enzyme deoxyribonuclease and its distribution in fungi has received limited attention and most of the data have been obtained from *Neurospora crassa* (Zamenhof and Chargaff, 1949).

The second phase of nucleic acid degradation involving dephosphorylation of nucleotides has also been reported in a few fungi (Laskowski, 1951). Casida and Knight (1954) found that a phosphatase extractable from *Penicillium chrysogenum* caused dephosphorylation of riboflavin monophosphate. Similarly phosphatases obtained from *Aspergillus niger* (Malmgreer, 1952; Mann, 1944 a; Krishnan and Bajaj, 1953 a) as well as *Penicillium chrysogenum* (Krishnan, 1951 b, 1952; Sadasivan, 1950) could hydrolyze adenosine phosphates. Kaplan *et al.* (1951) obtained a specific diphosphopyridine nucleotidase, which could split off nicotinamide from the dinucleotide leaving adenosine diphosphate ribose.

A few scattered reports on deaminating enzymes affecting the nucleotides and nucleosides are also on record. An enzyme adenylic deaminase was reported by Kaplan *et al.* (1952) from *Aspergillus oryzae*, which could deaminate adenosine, adenosine monophosphates (AMP), adenosine diphosphate (ADP) as well as nicotinamide adenine dinucleotide (NAD).

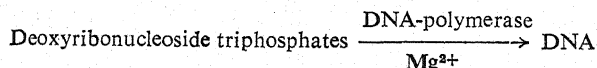
Reports on catabolism of the free nitrogen bases-purines and pyrimidines, are also meagre, but it is generally believed that the overall processes of their breakdown are similar to those described above for animal system.

### Biosynthesis of Nucleic Acids

As already indicated, nucleotides are the building blocks of nucleic acids and therefore, their activated forms (nucleoside triphosphate) along with the appropriate enzyme and cofactors are the primary requirements of nucleic acid synthesis. Moreover, cellular synthesis of nucleic acid requires exact informations about the order of the nitrogen bases in the polymer, *i.e.* it needs a template.

Available informations indicate that there exist two separate pools of free nucleotides in most of the fungal cells (Zalokar, 1965). This was shown in *Candida* and *Neurospora* (Cowie and Bolton, 1957; Zalokar, 1962). The nucleotides generally occur as phosphates of adenine, uridine etc. and are found accumulated and precipitated in vacuoles (Svihla *et al.* 1963). These cellular nucleotides may be utilized for biosynthesis of nucleic acid or in intermediary metabolism.

**Synthesis of DNA.** DNA has only been found in nuclei and replication of DNA is a must preceding a cell-division. DNA-replication has formed the text of many earlier reviews (Cavalien and Rosenberg, 1962; Grunberg-Manago, 1962; Bronk, 1973). The general process involved may be schematized as below:



The enzyme DNA-polymerase discovered by Kornberg (1960), converts four deoxyribonucleoside 5'-triphosphates into DNA with liberation of one pyrophosphate molecule per nucleotide. However, this enzyme has not been detected in fungi, although an enzyme separated by Grunberg-Manago and Wisniewski (1957) from yeast synthesizes polynucleotides. Synthesis of DNA in the slime mold *Physarum* occurs 1-2 hours after nuclear division which is followed by a resting period of about 12-20 hours. In contrast, RNA synthesis proceeds in a continuous manner in yeast also.

**RNA Synthesis.** The site for RNA synthesis is also the nucleus which is subsequently released to the cytoplasm. Indication to that effect was noted by Caspersson (1950) who found RNA near the nuclei in yeast, but later the use of radioactive tracers have provided direct evidences regarding RNA synthesis in nuclei. Zalokar (1960 b) demonstrated RNA synthesis in fungal nuclei by the use of centrifuged cells. Newly labelled RNA polymers appeared exclusively in nuclei

during the first 4 minutes after the fungal cells were provided with  $^{14}\text{C}$ -adenine,  $^3\text{H}$ -uridine, or  $^3\text{H}$ -cytidine; immediately before centrifugation. The origin of different types of RNA, viz. transfer-RNA, ribosomal-RNA, and messenger-RNA is however, still uncertain, particularly that of ribosomal RNA. Evidences obtained from yeast (Ycas and Vincent, 1960) and *Neurospora* (Schulman and Bonner, 1962) indicate strongly that in fungi messenger RNA is made by DNA. The origin of transfer RNA is also indicated to be formed by DNA (Yanofsky *et al.*, 1961), but that of ribosomal RNA, which in other cells is thought to be synthesized in nucleoli, is yet to be ascertained in fungi. The overall process of synthesis of RNA is similar to that of DNA replication and requires the enzyme RNA-polymerase, magnesium ions, and ribonucleotides in the triphosphate form. Above all it also requires a template, which is usually provided by the chromosomal DNA.

*Synthesis of Nucleotides.* Nucleotides, the building blocks of nucleic acids are known to be synthesized in all types of cells from ammonia, carbon dioxide, amino acids and ribose phosphate. The complete synthetic pathways of either purine or pyrimidine nucleotide in fungi are yet to be elucidated.

*Pyrimidine Synthesis.* Biosynthesis of uridylic acid (Uridine 5'-phosphate, UMP) requires six enzymes with carbamyl phosphate as the starting product. The enzymes involved are carbamyl phosphate synthetase (CPSase) catalysing the synthesis of carbamyl phosphate from glutamin or ammonium. The other five enzymes are (i) aspartate transcarbamylase (ATCase), dihydroorotase (DHOase), dihydroorotate dehydrogenase (DHO dehas), orotidylate pyrophosphatase (OMP-ppase) and orotidylate decarboxylase (OMP decase). The sequence of reactions of uridylic acid synthesis is shown in Fig. 10.13.

During the last decade, this aspect has been extensively investigated in eukaryotic cells including those of fungi and it seems possible that in fungi all the six enzymes of the pathway may be a single, rather fragile complex *in vivo*. The pathway of pyrimidine biosynthesis also seems to follow slightly different pattern, as in fungi a complete separation of the arginine and pyrimidine biosynthetic pathways is possible, because for each pathway, carbamyl phosphate (CAP) can be derived from a specific carbamyl phosphate synthetase. However, in yeast these two pathways share CAP pools, and in fact

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CAP can flow one "pool" to the other. The existence of enzyme-complex in fungi is another interesting feature. A complex of the first

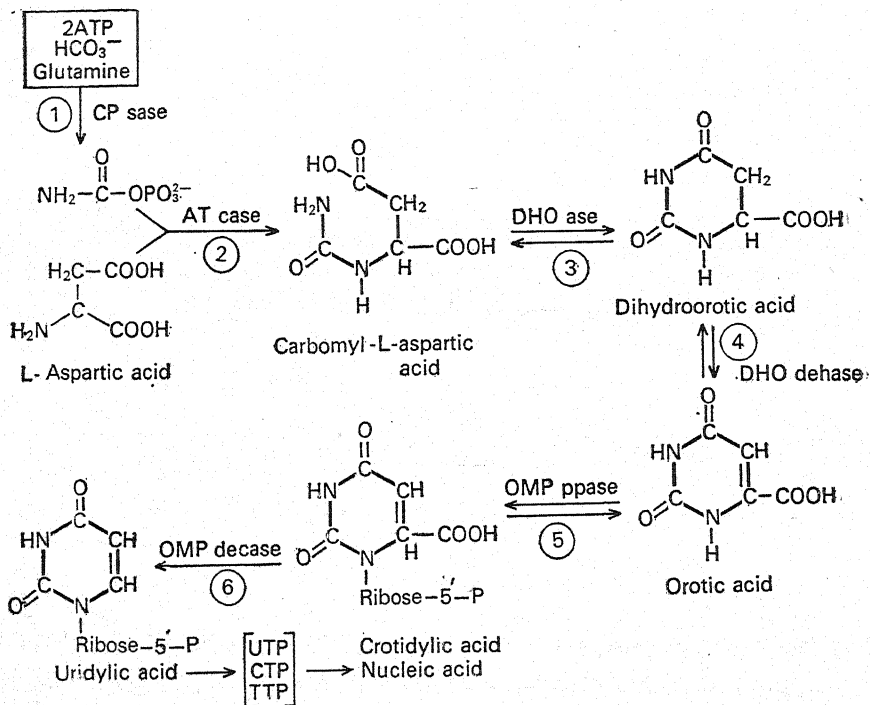


Fig. 10.13. Enzymic reactions of uridylic acid biosynthetic pathway.

two enzymes, namely CPSase-ATCase is also indicated from genetical studies. These two enzymes are coded for by a single polar gene. Recently, Mary (1972) has presented a comprehensive account of regulation and genetic control of uridylic acid biosynthesis in eukaryotic cells including those of fungi. Genes coding for different enzymes of the pyrimidine pathway have been identified. A single polar structural gene, *pyr-3* in *Neurospora* and *Ura-2* in yeast codes for both CPSase and ATCase. The rest of the genes for UMP biosynthesis are unlinked.

*Purine Synthesis.* Investigations with adenineless mutants of *Neurospora crassa* led Mitchell and Houlahan (1946) to propose the following sequence of reactions for adenosine synthesis.

"X"

Inosine — Adenosine

Further evidences from mutants of *Ophiostoma multiannulatum* (Fries, 1947, 1949 a; Fries *et al.*, 1949), *Ustilago zae* (Perkins, 1949) and *N. crassa* (Pierce and Loring, 1945) have also supported the above scheme, and it seems that the overall process of purine synthesis in fungi is identical to that operating in animal systems.

## UTILIZATION AND METABOLISM OF INORGANIC SUBSTANCES

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The role of minerals in the nutrition of fungi started receiving attention with the classical researches of Raulin (1869), who concluded that the optimum growth of the fungus *Aspergillus niger* on a synthetic medium required the incorporation of several minerals like magnesium, potassium, zinc, iron etc. Omission of any of these elements resulted in reduced growth of the fungus. The pioneering effort of Raulin was supplemented with a series of investigations like those of Naegeli (1880), Benecke (1895), Molisch (1892, 1894) and Wehmer (1895). In line with the investigations on higher plants, various mineral nutrient solutions, like Pfeffer's solution, Richard's solution, Uschinsky's solution, Czapek's solution etc. were employed in studies with fungi also. All these nutritional investigations were marked with serious methodological limitations and often led to erroneous conclusions like the theory of chemical stimulation, propounded by Pfeffer (1895). Foster (1949) has given a detailed historical account of the earlier theories and concepts regarding mineral nutrition of fungi. Mention may be made here of the investigations initiated by Steinberg (1919 b), which led to a complete shift in our earlier concept of the mineral elements from spurious "stimulants", to biologically essential nutrients for fungi. The change brought about by Steinberg was not merely confined to the concept but was extended to the methodology also, and the (Steinberg 1937) as well as Richards and Troutman (1940) employed spectroscopy for the analysis of the fungal ash-samples, which enabled the detection of a host of other elements in mycelial residues.

As per our present concept, fungi like other organisms, exhibit two types of mineral requirements, viz. (i) those which are required in comparatively larger amount, and (ii) those required only in tra-



ces. The macroelements (belonging to the first category) required by fungi include phosphorus, potassium, sulphur and magnesium; while micronutrients (trace elements include iron, zinc, copper, manganese and molybdenum. However, use of such terminology like micro and macro elements, trace elements, minor elements etc. seems to be of little significance, because the amount of a particular element is no index or measure of its essentiality. Necessity of cobalt in the synthesis of vitamin B<sub>12</sub> (cyanocobalamin) has been demonstrated, but its positive role has been little understood. In fact, the mechanism, by which micro-elements play a part in fungal nutrition is more or less obscure (Tandon and Agarwala, 1956), though, a few of them are known to form an integral part of several enzymatic systems, or a constituent of certain pigments. Obviously, more attention is required to unravel their possible role in metabolic processes, which hold greater promise to-day in view of better availability of purer chemicals and neutral glasswares, as well as the sophistication attained in various physical and biochemical methods.

### NON-METALLIC MINERALS

The fungal hyphae are mainly (95%) composed of non-metallic elements, like hydrogen, oxygen, carbon, nitrogen, sulphur and phosphorus, although a few fungi accumulate arsenic, selenium, chlorine, silicon and other non-metals. Due treatment to the role of carbon and nitrogen in fungal nutrition has been detailed in earlier chapters. Role of other have been discussed here, with emphasis clearly lying over sulphur and phosphorus which are available to fungi as minerals.

#### Hydrogen

Hydrogen which is the most abundant element in the whole of universe, occupies a similar position in the fungal cell also. It goes into the composition of nearly all the organic components of the fungal cell including those of physiological significance and thus is of both structural and functional importance to fungi. Hydrogen content is generally expressed in terms of weight per cent, although it does not indicate its relative abundance in the cell. Hydrogen-content of dry cells is seldom recorded. Hilpert *et al.* (1937) found that mycelium of *Aspergillus niger* contained 6.7% hydrogen, and it is considered that on an average the hydrogen-content of fungal cells in general lies between 6 and 8%.



Unlike some hydrogen bacteria, fungi are unable to utilize elemental hydrogen, and depend entirely on hydrogen containing compounds for their hydrogen requirement. Obviously, water is the most abundant source of hydrogen for fungi, as for other living beings. Water generally ionizes into hydrogen ( $H^+$ ) and hydroxyl ( $OH^-$ ) ions, both of which exert immense influence on biological processes. Water-content of fungal mycelium is generally higher than that of spores. Gortner (1949) differentiated two kinds of cellular water, viz. "free" and "bound" water. It is the free water which acts as the solvent and helps in translocation of metabolites. It may be removed by drying at room temperature in a desiccator. Bound water remains absorbed by the protoplasm and can only be removed by drying at elevated temperatures (80-110°C). Also, at low temperatures (below 4°C) bound water does not freeze, which enables the cells particularly spores to withstand freezing temperatures. The quantity of bound water in a fungus cell may vary according to environmental conditions. Todd and Levitt (1951) found that bound water content of *A. niger* mycelium increased with the increasing concentration of glucose.

### Oxygen

Fungi display a wide pattern of response to free oxygen, although none of them is apparently obligate anaerobe. According to their response to oxygen, fungi may be classified into three types, viz. (i) Aerobic fungi, needing uncombined oxygen, (ii) facultatively anaerobic fungi, which do not need free oxygen but can use it both in free and combined forms, and (iii) microaerophilic fungi, which can grow in traces of free oxygen, i.e. at low oxygen tension. Oxygen-requirement of aerobic fungi also varies with the species. *Aspergillus oryzae* shows maximum respiration-rate, when the partial pressure of oxygen is 500 to 630 mm Hg. In *Ascophanus carneus*, effect of reduced oxygen-pressure was studied by Ternetz (1900), who recorded that the mycelium which could hardly grow at 10 mm Hg., exhibited better growth response with increasing oxygen pressure, and the growth attained at 120 to 140 mm Hg was better than at the atmospheric pressure. Little difficulty is encountered while growing an aerobic or facultatively aerobic fungus in small tubes or flasks with regard to its oxygen requirements. However, when aerobic fungi are to be grown in large quantities, it becomes necessary to provide for the proper aeration of the culture. Ordinarily,

this is achieved by increasing the exposure of the medium to the atmosphere by dispensing it in shallow layers in suitable containers, e.g. Fernbach flask, Kolle flask etc. However, sometimes it is more advantageous to provide increased aeration to the culture by constantly shaking the inoculated liquid medium or by passing a stream of sterile air through the culture-solution during incubation. In stationary liquid cultures there is an unequal aeration of the aerial and submerged parts of the fungus. Tamiya (1942) demonstrated that in a stationary liquid culture of *Aspergillus oryzae*, the enzyme systems of the submerged mycelium are more easily poisoned by cyanide than are those of aerial mycelium. Besides, it is a common observation that in stationary liquid cultures, only the aerial hyphae sporulate, and not the submerged ones. Production of penicillin and other antibiotics are greatly enhanced due to constant aeration of the culture.

Microaerophilic fungi are represented only by a few soil or aquatic fungi and they have the capacity to grow in only traces of oxygen. Such adaptation is important with respect to their survival. Hollis (1948) reported that *Fusarium oxysporum* was able to survive for 13 weeks in almost anaerobic condition with some abnormalities in its mycelium. The same conditions, however, resulted in the death of *F. eumartii* within 3 weeks. Recently, Held (1970) reported that the aquatic phycomycete *Aqualinderella fermentans* appears to have lost its requirement for oxygen as well as its capacity for respiration, through regressive evolution, in adaptation to an obligatory fermentative existence in an environment poor in  $O_2$  and rich in  $CO_2$  (Emerson and Held, 1969; Held *et al.*, 1969).

In laboratory, conditions reduced oxygen tension is achieved by (i) mechanical removal of oxygen from the culture vessel and subsequent replacement with nitrogen, helium, or a mixture of nitrogen and carbon dioxide or (ii) addition of a reducing compound such as sodium thioglycolate to the medium, in quantity just insufficient to create anaerobic condition (This method is commonly employed in culture of anaerobic bacteria).

Oxygen is required by living cells for respiration. Wieland (1932) formulated that all biological oxidations took place by removal or transfer of electrons from reduced donors to an appropriate acceptor. This concept got support from the widespread occurrence of microbial growth under strictly anaerobic conditions and it was never in

doubt until Mason (1955) by the use of isotopic  $^{18}\text{O}_2$  demonstrated that in biological oxidation direct incorporation of molecular oxygen is also possible. Thus we came back to the original ideas of Lavoisier and Priestley, that biological oxidation is often brought about directly by the addition of molecular oxygen. It is now known that such direct incorporation of molecular oxygen into an organic molecule is catalyzed by some specific enzymes, which are collectively designated as oxygenases. Various types of oxygenases take part in the oxidative phase of respiration. A full account of terminal oxidation, and the enzymes which help to activate and assimilate oxygen, alongwith the electron-transport chain has been given separately in chapter IX. Obviously in microbes, the most important role of oxygen is that of terminal electron acceptor.

Presence or absence of oxygen may have its effect both on growth yield and metabolic activities of the microorganisms. Such effects are rather simple and absolute in case of obligate aerobes or anaerobes, but in case of facultative organisms the situation becomes complicated. However, growth yield depends possibly upon ATP-yield and also upon whether in facultative organisms, the aerobic or anaerobic pathways of ATP generation are utilized. It has been observed that facultative organisms attain appreciably better growth under aerobic conditions. Metabolic influence of oxygen has also been observed but quantitative data are insufficient. The wide range of fermentation products, like organic acids and alcohols by organisms growing under low oxygen tension, indicates the need for terminal electron acceptors to balance oxidation reactions connected with utilization of reduced organic substrates for growth. These are oxidized completely to  $\text{CO}_2$  and water, if the oxygen availability is not growth-limiting. However, some fungi seem to require high partial pressure of oxygen for optimum production of some of their secondary metabolites, *e.g.* penicillin (Bartholomew, 1960; Hirose *et al.*, 1967). Oxygen also exerts regulatory influence on TCA cycle, aerobic conditions favouring enhanced synthesis of all TCA-cycle-enzymes, with a corresponding decline in glycolytic enzymes. Enzymes of respiratory chain as well as individual electron transport components are also affected by oxygen concentrations. Such effects are more obvious in oxygen limited growth of facultative organisms. Thus, oxygen's influence is not limited to the enzymes which utilize oxygen, such as the succinate oxidase system but extends to some associated enzyme systems also, like those of glycolysis and TCA

cycle. We may thus conclude that both the Pasteur and Crabtree effect operate at this level.

### Phosphorus

This non-metallic element is essential for all forms of life including fungi. Raulin (1869) recognised the essentiality of phosphorus in the nutrition of *Aspergillus niger*. Lafar (1904) stated that the indispensable nature of phosphorus for the growth and metabolic activities of fungi is a well and long recognised fact. Brown (1925) reported that phosphates influenced the mycelial growth in various *Fusaria* he studied. The metabolic effect of phosphorus deficiency was indicated quite early by Cockefair (1931), who suggested that sugars could not be oxidized and nitrates could not be reduced to amino acids without an adequate supply of phosphorus. Chemical analysis of fungal structures has always indicated high phosphorus content in the fungal ash. Even definite compounds containing phosphorus have been isolated from fungi, which obviously indicates that phosphorus is also a structural component of fungus cell. The phosphorus content of fungal mycelium may vary according to the age, as well as the availability of this element. Rennerfelt (1934) in *Aspergillus niger* found that the younger mycelium of this fungus had a higher phosphorus content than the older mycelium. Also, by increasing the concentration of phosphate in the culture medium, the mycelial phosphorus content could be increased. Spores contain higher amounts of phosphorus than the mycelium. However, their inorganic as well as organic phosphates tend to leach out easily, as was reported by Bajaj *et al.* (1954) in *A. niger*.

Fungi apparently utilize phosphorus in the form of phosphate, although different phosphates support different amount of growth, even when the total quantity of phosphorus supplied to the fungus is maintained at the same level. Both inorganic as well as organic phosphates (esters) may be utilized by fungi for meeting their phosphorus requirements. Inorganic phosphates are generally furnished to the fungi in the form of their potassium salt, and most of the potassium phosphates like potassium orthophosphate ( $K_3PO_4$ ), potassium metaphosphate ( $KPO_3$ ), potassium pyrophosphate ( $K_4P_2O_7$ ), potassium monohydrogen phosphate ( $K_2HPO_4$ ) and potassium dihydrogen phosphate ( $KH_2PO_4$ ) have been found to be generally utilizable by fungi. Potassium monohydrogen and dihydrogen phosphates are more frequently incorporated in culture media, because besides furnishing utiliz-

able phosphate and potassium ions, these salts also act as useful buffers and exert a controlling influence over the pH-changes in the medium caused by fungal growth and metabolism. Dox (1911-1912) studied in detail the phosphorus utilization by *Aspergillus niger* and found that besides these inorganic phosphates some phosphorus containing organic compounds like phytin, sodium glycerophosphate, sodium nucleinate, casein and ovovitellin, also furnished utilizable phosphorus to the fungus and supported excellent growth. However, sodium phosphate ( $\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$ ) and sodium hypophosphite ( $\text{Na}_2\text{H}_2\text{PO}_2 \cdot \text{H}_2\text{O}$ ) were unutilizable and were apparently toxic to the fungus. Different fungal species also exhibit differences in their growth response on different phosphorus sources. *Merulius lacrymans* in a glucose containing medium attained better growth when furnished with inorganic phosphate, while *Marasmius chordalis* preferred organic phosphorus (Smith, 1949). However, on another carbon source, viz. cellobiose, *M. lacrymans* exhibited better affinity for organic source of phosphorus. Utilization of both inorganic and organic phosphates by fungi involves their enzymatic hydrolysis. The concerned enzymes, phosphatases have been recorded in a number of fungi, which have been adequately listed by Cochrane (1958). Both acid and alkaline phosphatases have been recorded among fungi.

Fungi also exhibit varying requirements of phosphorus. Some fungi like *Brevilegnia gracilis* (Bhargava, 1945), *Curvularia lunata* (Srivastava, 1951), *Fusarium* spp. (Brown, 1925; Agarwal, 1957; Jaurihar and Mehta, 1972) *Aspergillus terreus* (Mehrotra and Tandon, 1970) *Curvularia* spp. (Agarwal, 1958 a; Singh and Tandon, 1969), *Alternaria tenuis* (Singh and Tandon, 1967 a, b) etc. are atleast able to make feeble growth on phosphorus-deficient media. Others, like *Achlya* sp., *Isoachlya anisospora* and *Saprolegnia* sp., (Bhargava, 1945), and *Fusarium solani*, *Botryodiplodia ananassae* and *Macrophomina phaseoli* (Bhargava and Tandon, 1963) do not grow at all in the absence of phosphorus. Fungi also appear to differ in their quantitative requirements of phosphorus. Schade (1940) found that the growth of *Leptomitius lacteus* was inhibited by potassium monobasic phosphate at concentrations above 0.003M. However, the isolate used by Dorries and Haase (1930) could tolerate much higher concentrations i.e. upto 0.066M, of the same phosphorus source. *Aspergillus niger* also required higher concentration of phosphate in the medium, as Mann (1944) observed that with 0.002% or less of  $\text{K}_2\text{HPO}_4$ , the fungus attained only poor growth. When the  $\text{K}_2\text{HPO}_4$  concentration

was gradually increased upto 5% (equivalent to 0.089%P), the fungal growth recorded corresponding increase. Agarwal (1957) found that *Fusarium coeruleum* could utilize much higher concentration of phosphate.  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  were tolerated upto the concentrations of 0.2051% and 0.2507% of P respectively. However, when furnished with  $\text{K}_3\text{PO}_4$ , stimulatory effect on growth was observed upto 0.0684% P only. Beyond this concentration, the fungal growth was inhibited. Jaurihar and Mehta (1972) recorded best mycelial growth and sporulation of *Fusarium moniliforme* with potassium monohydrogen phosphate at concentration 0.512p/100 ml equivalent to 0.0912%P. Mehrotra and Tandon (1970) found that for *Aspergillus terreus* strains,  $\text{Na}_2\text{HPO}_4$  at concentrations between 0.2 to 0.3 g/l was the most suitable both for mycelial growth as well as itaconic acid synthesis. Higher concentration did support better mycelial output, but acid-production was checked.

Phosphorus plays some vital metabolic roles, some of which are known for long. As a constituent of phospholipids, which take part in the formation of cell-membranes, phosphorus exerts regulatory influence over cell permeability. This non-metallic mineral is also a part of nucleoproteins, which are important functional component of the protoplasm in general and the nuclei in particular. In their capacity as buffers, phosphates largely regulate the pH of the protoplasm. A number of enzymes and coenzymes contain phosphorus as one of their important constituents. Owing to its diverse roles in fungal metabolism, availability of phosphorus obviously affects the fungal growth and activities at various levels. An attempt to record the possible discrepancies in the fungal metabolism due to phosphorus deficiency was made by Mann (1944, 1944 a), who could observe that phosphorus-availability had definite bearings upon some vital processes like nitrogen assimilation, vitamin synthesis, oxygen-consumption, etc., besides the overall growth of *Aspergillus niger*. Phosphorus-assimilation in turn is also affected by the availability of oxygen. This indicates that respiration and phosphorus metabolism are closely related processes. Inhibitory influence of some respiratory inhibitors, like iodoacetate, azide and cyanide on phosphorus metabolism also supports this concept. It is now known that phosphorus participates in most of the steps of EM pathway either in the form of substrate or enzyme or coenzyme. Higher concentrations of  $\text{P}_i$  is now considered to stimulate EM pathway and inhibit HMP pathway (cf. D' Adamo, 1963). Kravitz and Guarino (1958) demonstrated such



effects of Pi in cell-extract, and it is believed that such a role of Pi is due to its functioning both as a substrate in the triose-P dehydrogenase reaction and as an inhibitor of the glucose-6-P dehydrogenase and the transaldolase reactions. However, what will be the intensity of such effects in living cells is not yet confirmed.

Role of Pi in energy transfer during terminal electron transfer and oxidative phosphorylation is now well recognised. The electrons removed at the four dehydrogenation steps of TCA cycle and accepted by NAD and FAD, are transferred through a terminal respiratory chain of cytochromes to final hydrogen acceptor, the oxygen. During this transfer of electrons, changes in free energy contents take place, and the energy so released is utilized in the synthesis of ATP from ADP and Pi. The ATP molecules then serve as store-houses of chemical energy and are the principal cell components functioning in energy-transfer within the cell.

### Sulphur

It was observed by many earlier workers that fungi growing on sulphur-deficient media either attained feeble growth or failed to thrive (Naegeli, 1880; Rabinovitzsereni, 1933 Schade, 1940; Steinberg, 1941; Bhargava, 1945; Grewal, 1954; Agarwal, 1957; Tandon M.P., 1950; Tandon and Bilgrami, 1958; Saksena and Sarbhoy, 1960; Tandon, 1961; Haglund and King, 1962). Moreover, the ability of fungal organisms to utilize various sulphur containing nutrients was also recognised (Armstrong, 1921; Volkonsky, 1933 and Saksena *et al.* 1953). Further studies have supplemented these data, and it is now well known that sulphur plays a significant role in the metabolic activities of fungi. It is known to be essential for the biosynthesis of sulphur-containing amino-acids, besides being a component of the sulphahydryl or thiol group of many enzymes (Lilly and Barnett, 1951; Cochrane, 1958; Tandon, 1961; Bhargava and Tandon, 1963), coenzymes and vitamins, which in turn affect various other vital processes of fungi.

Commensurate with their general nutritional behaviour, fungi exhibit variations in their choice and ability to utilize different sources of sulphur also (Hawker, 1950; Lilly and Barnett, 1951). On the basis of data obtained by Lwoff (1932) and Volkonsky (1933 a), Fischer distinguished two groups of microorganisms, viz. (i) Enthiotrophic, including those able to utilize sulphate sulphur and (ii) Parathiotrophic including those which could not utilize sulphate ions but could

use reduced sulphur. The parathiotrophic fungi have been further divided into two groups including (i) those requiring a sulphur-containing amino acid and (ii) those utilizing inorganic reduced sulphur compounds as well as reduced sulphur containing organic compounds (Lilly, 1965). Steinberg (1936, 1941) concluded that those inorganic sulphur compounds, which contained oxidized sulphur were utilized by *Aspergillus niger*, while sulphides and disulphide were unutilizable. Among the organic sulphur-sources, alkyl sulphonates and sulphinates were excellent S-sources, while the alkyl thioalcohols, sulphides and disulphides were unutilizable S-sources.

Fungi, lacking in the ability to utilize 6-valent sulphur ( $\text{SO}_4^{-2}$ ), or which may utilize organic sulphur-source to greater advantage, mostly belong to the aquatic phycomycetous tax. Most of the Saprolegniales (Volkonsky, 1933, 1934; Leonian and Lilly, 1938; Bhargava, 1945; Ram Dayal, 1961; Davey and Papavizas, 1962) and all the known members of Blastocladales (Cantino, 1950, 1955) have been reported to be parathiotrophic. *Blastocladia pringsheimii* requires methionine, and *B. ramosa* utilizes methionine, cystine and cysteine (Crasemann, 1957). They could not utilize sulphate sulphur, although thioacetamide could support-traces of growth. It seems interesting that all those genera, which grow in an aquatic habitat marked with lower oxygen availability or even anaerobic conditions, are dependent upon reduced sulphur, but whether this has got any ecological or biochemical relevance is yet to be ascertained.

Many of the fungi appear to be more versatile as far as their ability to utilize sulphur sources are concerned. For instance, *Brevi-legnia gracilis* (Bhargava, 1945), *Pythium* spp. (Saksena *et al.*, 1953), *Penicillium chrysogenum* (Hockenhull, 1948), *Pestalotia malorum* (Tandon, 1950) etc. were able to utilize a variety of sulphur sources, including inorganic and organic compounds like sulphides, thiosulphate, sulphur-containing amino acids, thiourea etc., besides the various oxidized forms of inorganic sulphur. Studies on sulphur requirements of several other fungi have also indicated that different fungi might respond differently to various sources of sulphur and that some might even exhibit specific choice for their sulphur-source. However, for most of the fungi sulphate sulphur appears to be the most favourable source (Lilly and Barnett 1951; Agarwal, 1958; Tandon and Bilgrami, 1958; Agarwal and Ganguli 1960; Bhargava and Tandon, 1963; Hasija, 1969; Mehrotra and Tandon 1970). Several fungi are also able to utilize elemental sulphur (Abbot, 1923; Mc-



Callan and Wilcoxon, 1931; Sciarini and Nord, 1943; Miller *et al.* 1953; Davey and Papavizas, 1962).

Available data on sulphur nutrition of fungi also indicate that different fungal organisms differ in their capacity to grow without a sulphur-source. Fungi like *Alternaria tenuis* (Rabinovitz-sereni, 1933), *Gloeosporium musarum* (Grewal, 1954), *Pestalotia malorum* (Tandon, 1950), *Fusarium coeruleum* (Agarwal, 1957), *Curvularia penniseti* (Agarwal, 1958 a), *Phyllosticta* spp. (Tandon and Bilgrami, 1958), *Pestalotia versicolor* (Agarwal and Ganguli, 1960), *Curvularia pallescens*, *Alternaria citri* and *A. tenuis* (Hasijsa, 1969) failed to grow in sulphur deficient media. However, an isolate of *A. tenuis* (Singh and Tandon, 1967) was able to make feeble growth even when sulphur was completely eliminated from the culture medium. Other fungi, which have been found to be able to survive in complete absence of sulphur include, *Aspergillus niger* (Steinberg, 1941), *Curvularia* spp. (Srivastava, 1951; Singh and Tandon, 1970), *Fusarium solani*, *Botryodiplodia ananassae* and *Macrophomina phaseoli* (Bhargava and Tandon, 1963), besides some Mucorales (Sarbhoj, 1965).

### Metabolism of Sulphur

Whatever be the source, the sulphur utilized by a fungus is incorporated into a variety of organic molecules, which enter into different components of the fungus cell. Several sulphur containing cell-constituents are known in fungi, including amino acids (cysteine, cystine, and methionine), vitamins (thiamine and biotin), cyclic choline sulphate, enzymes with sulphahydryl or thiol group, other proteins, the tripeptide glutathione, antibiotics like penicillin and gliotoxin etc., besides a number of miscellaneous products.

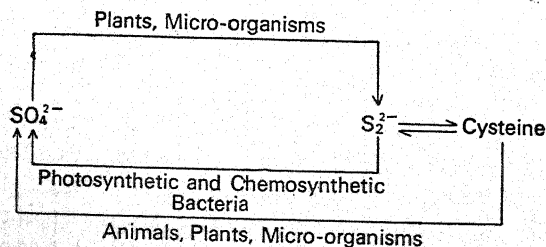


Fig. 11.1. Sulphur cycle.

However, till recently very little was known about the pathway of

sulphur metabolism in fungi, although Davis (1955) had proposed an outline of the possible steps for reduction of sulphate and subsequent transformation into an organic molecule. Investigations in the last fifteen years or so, particularly with biochemical mutants of some fungi and bacteria have yielded considerable information, which together with the large nutritional, genetic, and enzymic data have enabled us to trace out a more realistic pathway for sulphur metabolism in microorganisms in general and fungi in particular. These developments have been well reviewed by Wilson (1962), Thompson (1967), Nicholas (1967) and Trudinger (1969).

Sulphur metabolism involves both reductive and oxidative processes and together form a cyclic course (Fig. 11.1), generally referred to as the sulphur cycle. During the course of this cycle, an eight electron change leads to the reduction of sulphate into sulphide possibly through various intermediates, some of which are thought to be quite unstable, while others are difficult to detect because they enter into chemical combination with components of biological systems (Postgate, 1963). Subsequently, the sulphide may be transformed into sulphur-amino acids or may be reoxidized back into sulphate by certain specific bacteria. Similarly, sulphur-amino acids may be reoxidized back to sulphate to complete the turn of the cycle. Many of the intermediate steps of the cycle are yet to be unravelled and according to the present evidences, the fungi are capable only to carry on sulphate reduction leading to the biosynthesis of sulphur-amino acids.

Postgate (1959) has distinguished two types of biological sulphate reduction, including (i) assimilatory, *i.e.* small scale reductions of sulphate to sulphur-amino acids; and (ii) dissimilatory, *i.e.* large scale reduction of sulphate into sulphide, primarily as a part of energy-metabolism. Again, fungi appear to carry on only the first type, *i.e.* assimilatory sulphur metabolism, whereas the dissimilatory metabolism of sulphur seems to be restricted to some specialized obligately anaerobic bacteria. It may, however, be pointed out that the two types of sulphate reduction appear to differ only a little. In fact, both the pathways involve (i) activation of sulphate through the action of the enzyme sulphate adenylyltransferase, (ii) formation of sulphite (or a bound "sulphite"), and (iii) the "direct" reduction of sulphite into sulphide (except in a few cases) without the formation of stable intermediates. The sulphite reduction in both the cases is probably catalyzed by a single protein in presence of a suitable elec-

tron-donor. At enzymic level also, the similarity between the two pathways is being increasingly recognized. However, the little differences between the two pathways have been pointed out by Peck (1961, 1962). While assimilatory sulphate-reducing microbes require 3-phosphoadenylyl sulphate reductase for cysteine biosynthesis, the organisms, which carry on dissimilatory sulphur metabolism, need adenylyl sulphate reductase. For our limited purpose only assimilatory sulphate reductive pathway is considered here in detail.

### ASSIMILATORY SULPHATE REDUCTION

The pathway for assimilatory sulphate reduction has been elucidated as a result of investigation on fungi and bacteria alike and evidences indicate that similar pathways operate in a number of fungi and bacteria, which may be presented as follows:

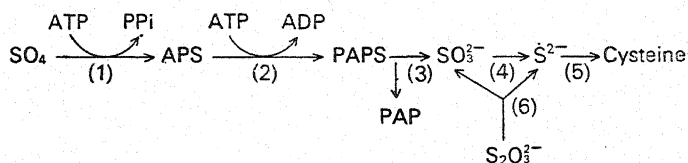
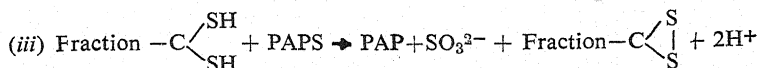
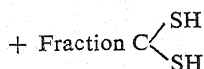
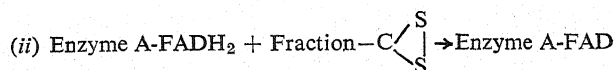


Fig. 11.2. Assimilatory sulphate reduction pathway, showing the main steps and the concerned enzymes: (1) sulphate adenylyltransferase, (2) Adenylyl sulphate Kinase, (3) 3'-phosphoadenylyl sulphate reductase, (4) sulphite reductase, (5) serine sulphydrase or O-acetylserine sulphydrase, (6) thiosulphate reductase.

Studies on the enzymes catalyzing sulphate activation and reduction have been reviewed by Wilson (1962). Isolation of cysteine requiring mutants of *Aspergillus nidulans*, *Neurospora crassa* and some bacteria, lacking in specific enzymes, e.g. sulphate adenylyl transferase, APS kinase, PAPS reductase, sulphite reductase, or O-acetylserine sulphydrase have provided sufficient evidence in support of this pathway. Evidences indicate that 3'-phosphoadenylyl-sulphate (PAPS) reduction in yeast requires  $\text{NADH}_2$  and FAD, two heat-stable enzymes A and B and a low-molecular-weight protein (fraction C) containing a functional disulphide group (Bandurski *et al.* 1960; Wilson *et al.* 1961; Asahi *et al.* 1961). Steps leading to PAPS reduction in yeast may be summarised as follows:





However, the sulphite ion formed in equation (iii) may not be in a free state. In fact, Torii and Bandurski (1964) have presented evidence that the reduction of PAPS in yeast does not yield free sulphite ion as the end-product, rather a protein bound sulphite ("bound sulphite") is produced. Later these two workers (Torii and Bandurski, 1967) were able to isolate this "bound sulphite", which they characterized as a sulphonyl group linked to protein possibly via a thiol group. However, investigations with isotopic  $^{35}\text{S}$  do not confirm a simple S-sulphonyl structure for the "bound sulphite". Although the structure of the protein fraction is not fully established, yet it showed approximation in its molecular weight to that of fraction C.

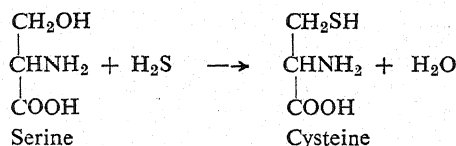
The next step of the assimilatory pathway accomplishes reduction of sulphite to sulphide, under the catalytic influence of sulphite reductase. Enzymatic extracts active against sulphite have been isolated from various biological systems including some fungi, like *Aspergillus nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae*. However, the mode of action of this enzyme, which affects a 6-electron change to produce sulphide as the end-product, without the formation of any apparent intermediate is still unknown. The electrons required for sulphite reduction are possibly supplied by  $\text{NADPH}_2$ , although sulphite reductases with electron donors other than  $\text{NADPH}_2$  are also known. Even the participation of an intermediate electron donor has been suggested (Siegel and Monty, 1964), e.g. reduced methylviologen (MVH), which links sulphite reduction with oxidation of  $\text{NADPH}_2$ . In the yeast *S. cerevisiae*, however, Niaki (1965) was able to distinguish between  $\text{NADPH}_2$ -linked and MVH-linked sulphite-reductase systems. However, some recent studies by Yoshimoto and Sato (1968 a, b) on sulphite reduction in yeast, have yielded a homogenous enzyme protein from a wild type yeast, which could bring about a complete reduction of sulphite into sulphide. Evidences indicate that this enzyme is a multicomponent flavoprotein containing flavine-adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which might be helping in the transfer of electrons from

NADPH<sub>2</sub> to sulphite. Besides some bacteria association of flavin compounds with purified sulphate reductase has also been reported in *Aspergillus nidulans* (Yoshimoto *et al.*, 1967). These flavin compounds have definite stimulatory action over NADPH<sub>2</sub>-linked sulphite reduction, as has been demonstrated in yeast (Prabhakar Rao and Nicholas, 1968), and *Neurospora crassa* (Leinweber and Monty, 1965; Siegel *et al.*, 1965).

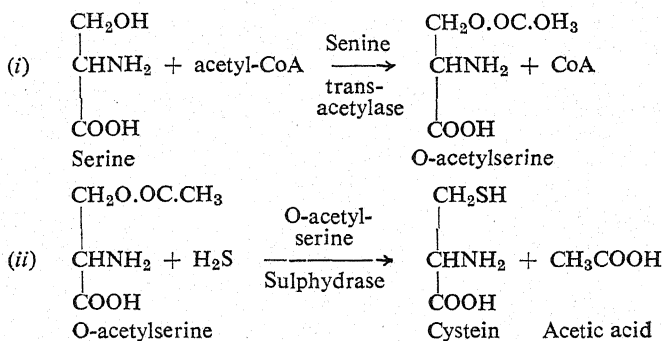
Another characteristic feature of sulphite reductase enzyme is the presence of a non-flavin chromophore, which has been shown in enzymes obtained from diverse organisms like *A. nidulans* (Yoshimoto *et al.*, 1967), yeast, bacteria and green plants. The exact chemical make-up of the chromophore is although uncertain as yet, there has been some indication that it may be a haemoprotein atleast in case of the enzyme obtained from spinach (Asada *et al.*, 1968) and *E. coli* (Kamin *et al.* 1968). Similar studies on chromophore of fungal sulphite reductases are still wanting and may be fruitful in the establishment of its exact chemical nature.

There has been some indication that reduction of sulphite into sulphide may, in addition to sulphite reductase system, require the enzymes A and B and fraction C of PAPS reductase system. Thompson (1967) suggested that enzymes of the PAPS reductase system might be acting through formation of "bound sulphite", because it is believed that sulphite must be bound to a thiol group before it can undergo reduction. Results obtained earlier by Wainwright (1961, 1962) supported these possibilities, because he could resolve sulphite reductase system of yeast into six active protein fractions, two of which were similar in properties to proteins A and C of PAPS reductase system.

The pathway of sulphate reduction is well understood upto this stage and evidences supporting the scheme upto this point abound. However, the final stages of the pathway leading to the biosynthesis of cysteine are not yet completely resolved, and infact have raised controversy. It has been suggested that different mechanisms may operate in different organisms or even in the same organism. However, this much seems certain that sulphide is the immediate precursor of cysteine-sulphur in a number of organisms. From *Saccharomyces cerevisiae*, Schlossman and Lynen (1951) were able to isolate an enzyme serine sulphydrase, which could bring about a condensation of sulphide and serine to produce cysteine. They suggested the following scheme for cystein biosynthesis in yeast.



Bruggemann *et al.* (1962) concluded that the enzyme serine sulphydrase has a wide distribution among fungi, bacteria and plants. However, Kredich and Tomkins (1966) proposed an alternative mechanism of cysteine biosynthesis in the bacterium *Salmonella typhimurium*, through the catalytic action of enzymes serine transacetylase and O-acetylserine sulphydrase :

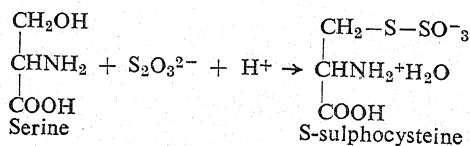


These two alternate mechanism of cystein-biosynthesis from sulphide may not only occur in different organisms, but even in the same organism. This kind of situation was reported in *E. coli*, where both serine sulphydrase (Bruggemann, *et al.*, 1962; Pasternak *et al.* 1965) as well as O-acetylserine sulphahydrase alongwith serine transacetylase enzymes were present. Comparison of specific activities of the two enzyme systems, however, indicated that in *E. coli* O-acetylserine sulphydrase is the main enzyme catalyzing the final stages of cysteine biosynthesis. Repression of O-acetylserine sulphydrase (Jones-Mortimer *et al.*, 1968) and of serine sulphydrase (Pasternak *et al.*, 1965) by the end-product cystein also suggests the same. Investigation into the distribution of O-acetylserine sulphydrase in various fungi studied so far may also yield interesting results, because the specific activity of the enzyme serine sulphydrase reported in those species are also of low order, as compared to the activity of the same enzyme in *E. coli*.

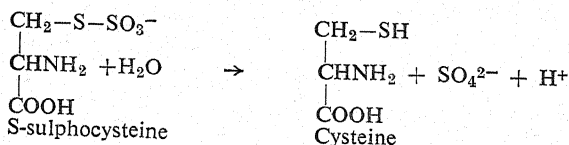
Similar controversy was raised regarding the role of thiosulphate in sulphate reduction, and it now appears that different conditions exist



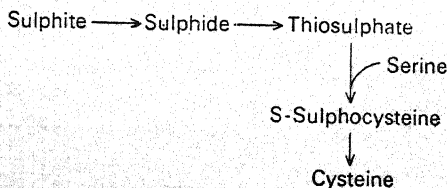
in different organisms. In *A. nidulans*, *E. coli* and *Salmonella typhimurium*, nutritional data indicated an intermediate role for thiosulphate in sulphite reduction (Wilson, 1962). Subsequent studies on a cysteine-requiring mutant of *A. nidulans* by Nakamura and Sato (1962, 1963 a) have strongly supported such a role of thiosulphate. This mutant accumulated S-sulphocysteine, when grown with sulphate. Isotopic studies with  $^{35}\text{S}$ -sulphate indicated that the accumulated S-sulphocysteine could not be derived from cysteine, and in fact, an enzyme catalyzing the formation of S-sulphocysteine through a different reaction was soon demonstrated in this fungus (Nakamura and Sato, 1963 b):



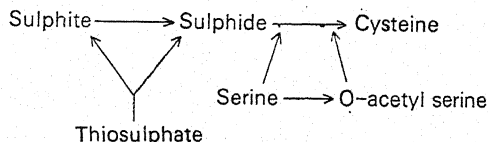
Nakamura and Sato (1960) had earlier indicated the presence of an enzyme, which could in yeast hydrolyze S-sulphocysteine into cysteine by the following equation:



Further studies with nutritional mutants (Yoshimoto *et al.* 1967) have yielded data which together with some earlier results indicate that at least in *A. nidulans* sulphide and S-sulphocysteine, both are intermediate products of cysteine biosynthesis, and that S-sulphocysteine is a derivative of sulphide possibly through thiosulphate. Thus the final stages of cysteine biosynthesis in *A. nidulans* may be represented as follows:



However, the possibility of an intermediate role of thiosulphate has been disputed in *Salmonella typhimurium* by Leinweber and Monty (1963). They have proposed a different hypothesis, according to which the thiosulphate molecule undergoes a reductive cleavage into sulphide and sulphite, before it is incorporated by the bacterium. Their contention gets strong support not only from studies with specific mutants of *S. typhimurium*, but also from the occurrence of enzymes catalysing direct reduction of thiosulphate to sulphide and sulphite in various organisms including *S. typhimurium* (Artman, 1956; Kawakami *et al.*, 1957; Kaji and McElroy, 1959; Woolfolk, 1962; Leinweber and Monty, 1963). Using  $^{35}\text{S}$ -labelled sulphate, Hiltz *et al.* (1959) concluded that in yeast also thiosulphate is not an intermediate compound in sulphate reduction. Thus the final steps of cysteine-biosynthesis in assimilatory sulphate reducing organisms other than *A. nidulans*, may be represented as under:



### Metabolic Control of Assimilatory Sulphate Reduction

As may be noted from the foregoing discussion, a good amount of evidences in support of the assimilatory sulphate reduction pathway has been obtained from inhibitory effects of cysteine either on the synthesis (repression) or on the function (feedback inhibition) of the enzymes involved in the pathway. Such controlling effects of cysteine over sulphate reductive pathway have been recorded in a number of organisms at different levels. Repression of sulphite reductase by cysteine has been recorded in *Neurospora crassa* (Metzenberg and Parson, 1966) and *Saccharomyces cerevisiae* (de Vito and Dreyfuss, 1964) besides several bacteria. Similarly, feed-back inhibition of sulphate adenyltransferase has been found to be caused by low concentration of ATP, PAPS and sulphide in yeast (Wilson *et al.*, 1961; De Vito and Dreyfuss, 1964). Inhibition of other enzyme functions due to cysteine has been recorded in several bacteria. In *Penicillium chrysogenum* cysteine, methionine and thiosulphate inhibit the uptake of sulphate.

Pasternak and his associates (1965) have made detailed studies on repression of enzymes of cysteine biosynthesis. They proposed that



enzymes of cysteine biosynthesis undergo "differential repression", and do not follow the principle formulated by Ames and Garry (1959) that all the enzymes of a biosynthetic pathway are repressed equally by the end-product. The hypothesis of Pasternak and his co-workers suggests that the repression of successive enzymes along the pathway of cysteine biosynthesis will require increasingly higher concentration of the end-product. Such a controlling and regulating effect of cysteine, helps in conserving the synthetic ability of the cell, since it avoids the synthesis of the enzymes not needed immediately by the cell.

## METALLIC MINERALS

The list of metallic elements known to be essential for fungi includes potassium, magnesium, iron, zinc, copper, calcium, gallium, manganese, molybdenum, vanadium, scandium, and cobalt. As indicated earlier, potassium and magnesium are included under essential macro-elements, while the rest of the metals are considered trace elements or micro-elements. However, nutritional roles of only a few of them have been studied in sufficiently large number of fungi and indeed mineral nutrition of only a few fungi has been studied in detail. Therefore, the generalisation that these minerals are essential for fungi in general seems to be more presumptive than established. As such, studies on mineral requirements of fungi need to be extended to as many taxa as possible. Moreover, investigation on this aspect also bears the possibility of enlarging the existing list of metallic minerals essential for fungi. The metallic requirements of fungi has not remained a field of fundamental research only, as of late it has assumed applied value also. In many microbe-based industries, use of appropriate trace-metals have literally led to commercial breakthrough in the production of antibiotics, vitamins, pigments, organic acids, and many other fermentation products. One may hope that such a commercial exploitation of our knowledge on this aspect may provide the necessary impetus to stimulate the research efforts now being made in this direction.

### Potassium

Among all the metallic minerals, potassium is present in the largest amount in both mycelium and spores, although concentrations as low as 0.001-0.004 M of this metal are adequate for most of the fungi (Steinberg, 1946; Jarvis and Johnson, 1950; Pisano *et al.*, 1954).

Usually, potassium is incorporated in the culture media in the form of phosphate and/or nitrate. The essentiality of this metal and its physiological role in fungal metabolism have been little investigated. Some fragmentary data relating to the effect of potassium on growth and metabolism of fungi indicate that sub-optimum level of this metal interferes with sugar utilization (Rennerfelt, 1934), and when completely absent, it causes increased accumulation of oxalic acid (Molliard, 1920; Rippel and Behr, 1934). *Mucor mandschuricus* accumulates pyruvic acid when the culture is lacking in thiamine but containing potassium or rubidium ions. In presence of thiamine and rubidium also pyruvic acid accumulates, but if thiamine and potassium are present together, no accumulation of pyruvic acid takes place. Earlier Muntz (1947) reported that potassium and ammonium ions exert stimulatory effect upon glucose-fermentative activity of yeast enzymic extracts. However, some enzymes show greater activity in preparations from potassium-deficient cells (Hofmann and Scheck, 1950). All these informations, though scanty, indicate that potassium has a possible role in carbohydrate metabolism. Data obtained from organisms other than filamentous fungi also indicate the same (Muntz, 1947; Orskov, 1948; Roberts *et al.*, 1949).

### Magnesium

The essentiality of magnesium for fungi was demonstrated quite early by Molisch (1892, 1895) and Benecke (1895), who found that magnesium could not be replaced by any other mineral element. Later, Steinberg (1946), Lavollay and Laborey (1938) and Nicholas and Fielding (1951) found that the growth of *Aspergillus niger* was proportional to the magnesium content of the medium. Usually magnesium is provided in the medium as sulphate at about 0.001 M concentration (24 mg/l). However, different fungi are able to tolerate even higher concentrations of this metal. Nicholas and Fielding (1951) noted that *A. niger* attained best mycelial growth, at 20 mg/l of magnesium. In *Allomyces arbuscula* only 9 mg/l of magnesium was sufficient for maximum growth of the fungus, but 200 mg/l was toxic (Ingraham and Emerson, 1954). In contrast, *Phycomyces blakesleeana* tolerated as high as 820 mg/l of magnesium concentration without any apparent inhibitory effect on growth although it caused a reduction in the carotene synthesis (Vail and Lilly, 1961).

*Penicillium glaucum*, *Botrytis cinerea* and *Alternaria tenuis* which were not able to grow without magnesium (Rabinovitz-Sereni, 1933), could tolerate without any harmful effect increasing magnesium concentration till the medium contained about 40% magnesium sulphate. Besides its concentration in the medium, various other factors are known to influence its incorporation into the fungal mycelium. Rippel and Behr (1930) concluded that age of the culture and nitrogen-source (ammonium or nitrate) influenced the magnesium intake by *A. niger*. Other factors influencing magnesium availability to the fungal cell include concentration of the carbon source (Steinberg and Bowling, 1939), concentration of other ions antagonistic to magnesium, and production of hydroxy acids or other chelate-forming compounds by the organism. Even the cultural condition may determine the availability of this metal; *Aspergillus terreus* shows a higher utilization of magnesium in surface culture than in shaken culture (Lockwood and Reeves, 1945; Lockwood and Nelson, 1946). Similarly, magnesium absorption is slower at neutral pH than at acid pH, and hence the optimum at neutrality is higher (Steinberg, 1945 a). At alkaline pH, in the presence of ammonium ion, magnesium uptake is impaired as it gets precipitated (Fries, 1945).

Role of magnesium in fungal metabolism is chiefly through its activating influence over various enzyme systems, including those of fermentation (Sumner and Somers, 1947; Malmstrom, 1953 a, b). An indirect evidence for its role in oxidative metabolism of carbohydrates may be derived from the reported enhancement of riboflavin synthesis due to magnesium deficiency in *A. niger* (Lavollay and Laborey, 1938, 1941; Sarasin, 1953), which obviously shows some interference with the normal oxidative pathway. Such enzyme activating effect has also been noted with manganese and other divalent ions, but magnesium is considered as the physiologically active metal (Lehninger, 1950; McElroy and Nason, 1954).

The phenomenon of ion-antagonism in the nutrition of fungi is well known (Foster, 1939, 1949; Perlman, 1949). Magnesium is also known to play its role in ion-antagonism against various toxic metals like mercury, boron, aluminium and copper (Lohrmann, 1940; Lockwood and Reeves, 1945; Marsh, 1945). Magnesium ions were found to antagonise the inhibitory effect of aluminum on itaconic acid synthesis by *A. terreus* (Lockwood and Reeves, 1945; Mehrotra and Tandon, 1970). MacLeod and Snell (1950) explained the phenomenon of ion-antagonism as resulting from competition of other

metals with magnesium for enzyme surfaces, and due to the inactivity or lower activity of the resulting metal-enzyme complex, as compared to the natural magnesium-enzyme complex. However, such competition may also take place at the cell-membrane, for instance absorption of copper by conidia of *Sclerotinia fruticola* is prevented by magnesium ions (Marsh, 1945). Horsfall (1957) suggests that the metal cations exert their toxic effect on fungi by acting at or very near the cell-surface and by replacing the non-toxic cations from the anionic surface sites on the cell-wall. Thus magnesium might be competing with copper for the anionic sites on the cell-wall and thereby preventing the absorption of copper ions. The chemical aspect of ion-antagonism has been adequately reviewed by Gortner (1949), who has concluded that the process of absorption is regulated by the relative concentration of different metallic ions.

### MICROELEMENTS

Fungal requirements of microelements have received much more attention than the macroelements. The vast amount of literature on this aspect has been reviewed by Foster (1939), Steinberg (1939), Perlman (1949). Hawker (1950), Lilly and Barnett (1951), and Cochran (1958). Many of the earlier reports presented conflicting data, which is obviously due to experimentation under less rigorous conditions. Investigations with trace metals have some inherent difficulties relating to non-availability of biologically pure chemicals and quality glass-ware, besides faulty inoculation methods. Some of these problems together with a few standard techniques including some biological methods for purification of culture media, have been described by various authors (Lilly and Barnett, 1951; Donald *et al.*, 1952; Nicholas, 1952; Sarswathi Devi, 1958) and it is now well realised that such studies need much more careful and rigorously controlled experimentation.

#### Iron

Raulin's (1869) conclusion that iron is indispensable for fungi was confirmed by Molisch (1892), Benecke (1895), Steinberg (1919), Roberg (1928) and many others. It is now firmly believed that iron is essential for all fungi, and it forms an integral part of the fungal protoplasm. In fact, association of iron with various enzymes including cytochromes, cytochrome oxidase, catalase and others have lent

so much support to the possible role of iron, that its essentiality has been taken as a forgone conclusion.

Iron requirement of most of the fungi may be met by furnishing only 0.1-0.3 ppm of iron, and some fungi are able to detect it even in much lower quantity. *Aspergillus niger* could respond to the presence of as little as 0.1  $\mu\text{g}$  iron in 60 ml of the medium (Nicholas, 1952). However, Tandon and Agarwala (1956) recorded that *Gloeosporium psidii* and *G. citricolum* required 25  $\mu\text{g}/\text{l}$  of iron to attain maximum mycelial growth as well as good sporulation, while a third species, viz., *G. limetticolum* needed still higher i.e., 50  $\mu\text{g}/\text{l}$  concentration of iron for best growth, while sporulation was best only at 5  $\mu\text{g}/\text{l}$  concentration. *Alternaria tenuis* attained best growth at 250  $\mu\text{g}/\text{l}$  of iron (Grewal, 1956). Tandon and Chandra (1962) recorded best growth of *Cercosporina ricinella*, *Colletotrichum gloeosporioides* and *Curvularia penniseti* at 0.2, 0.5, and 0.3  $\mu\text{g}/\text{ml}$  of iron respectively. Misra and Mahmood (1959) recorded similar response to iron in *Alternaria tenuis* and *Colletotrichum capsici*. In these studies, poor growth and sporulation were recorded when iron was either entirely lacking or when furnished in suboptimal quantity. Essentiality of iron has also been concluded in some species of *Fusarium* by Saraswathi Devi (1956, 1958) and in *Pyricularia oryzae* by Apparao *et al.* (1955). Steinberg (1950) recorded about 40-75 times increase in growth of certain fungi, when traces of iron were added to iron-free medium. Brian and Hemming (1950) reported the stimulatory effect of iron over sporulation of *Trichoderma viride*.

Iron may be furnished in either ferrous or ferric form because most fungi do not show any preference. *Pilobolus* however, requires chelated iron compounds (Page, 1962). pH of the culture media exerts marked influence over utilization of iron, at neutral and alkaline pH it becomes unavailable and is lost from the solution.

The most striking physiological effect of iron-deficiency is retarded growth and scanty sporulation. These effects are thought to be due to impaired synthesis of iron-containing enzymes under these conditions. A direct correlation between catalase production by *A. niger* and iron-content of the medium was shown as early as 1939 by Yoshimura (1939-1940). Similar effects of iron-deficiency has been observed on the synthesis of various enzyme systems of *Neurospora* by Nicholas and Goodman (1958). Deficiency of this metal depressed the synthesis of catalase, peroxidase, cytochrome c, cytochrome oxidase, DPNase as well as TPNH and DPNH diaphorases. Iron-deficiency

affects many other metabolic activities. Lewis (1944) found that synthesis of some vitamins by *Torulopsis utilis* was enhanced in media containing suboptimal amount of iron, while synthesis of a few other vitamins decreased. Organic acid fermentation (Foster, 1939; Perlman 1947, 1951), penicillin production (Knight and Frazier, 1945; Koffler *et al.*, 1947; Jarvis and Johnson, 1946, 1947), enhanced synthesis of certain pigments, like pulcherrimin in *Torulopsis pulcherima* (Roberts, 1946; Kluyver *et al.*, 1953; Cook and Slater, 1956) etc. are also affected by iron-content of the medium but in none of these processes, the mode of activity of iron is understood.

Studies on trace metal requirements of some soil Fusaria causing wilt diseases, by Sadasivan and his school have yielded quite interesting results (Sadasivan, 1967), which have opened the possibility of utilizing trace metals for controlling wilt diseases. Iron and manganese, particularly the former, were found to be quite effective in altering the potency of fusaric acid by preferential chelation *in vivo* in *Fusarium vasinfectum* infected cotton plants (Sadasivan, 1965). The trace metals were also found to influence the pectic enzyme systems of the pathogen *F. vasinfectum* (Subramanian, 1956). Iron in ferric form produced striking increase in the pectin methylesterase (PME) activity even at 0.25 ppm concentration, and thus appeared to be essential for PME production by this fungus. Iron also augmented polygalacturonase activity but only at the lowest concentration.

Role of iron as an effective ion-antagonist against certain toxic ions like copper and zinc was noticed during studies with *Pyricularia oryzae* (Apparao, 1959).

### Zinc

Essentiality of zinc for fungal growth was first recognised by Raulin (1896), and since then its necessity in fungal nutrition has been confirmed by various investigators (Steinberg, 1919; Roberg, 1928; Foster, 1939; Leonian and Lilly, 1940; Blank, 1941; Lindeberg, 1944; Ezekiel, 1945; Yogeshwari, 1948; Perlman, 1948; Chesters and Robinson, 1951; Tandon and Agarwala, 1956; Grewal, 1956; Saraswathi Devi, 1955, 1958; Agarwal, 1959; Tandon and Chandra, 1962; Sadasivan, 1965, 1967).

It is now almost established that all fungi require this metal, though as a micro-nutrient. In routine culture media zinc is added (as zinc sulphate) in concentrations between 0.5-1.0 ppm, although in some cases it may range between 0.001 to 0.5 ppm. Higher concen-



trations of this metal often prove toxic and may even lead to mutagenic changes in the fungal organisms. Genetically stable effects of higher levels of zinc have been recorded in *Fusarium* sp. (Dimock, 1936) as well as *Helminthosporium sativum* (Millikan, 1940). In sub-optimal concentration, also, zinc produces a multitude of metabolic effects, which is believed to be due to its role as an activator or a constituent of enzymes. A vast amount of literature has accumulated on this aspect, which have been reviewed from time to time (Foster, 1939, 1949; Perlman, 1949 b; Chesters and Robinson, 1951; Sadasivan, 1967). From available reports it appears that Zn-defici-

TABLE 11.1

SHOWING FUNGAL-PRODUCTS, WHOSE YIELD IS AFFECTED  
BY Zn-CONCENTRATION

Organisms	Product	Reference
<b>PRIMARY METABOLITES</b>		
1. <i>Aspergillus niger</i>	Citric acid	Shu and Johnson (1948) Clark <i>et al.</i> (1965)
2. <i>Candida guilliermondii</i>	Riboflavin	Tanner <i>et al.</i> (1945)
3. <i>Penicillium griseofulvum</i>	Myceliavamide	Bayan <i>et al.</i> (1962)
4. <i>Ustilago sphaerogena</i>	Coproporphyrin	Komai and Neilands (1968)
5. <i>U. sphaerogena</i>	Ferrichrome	Komai and Neilands (1968)
<b>SECONDARY METABOLITES</b>		
1. <i>Aspergillus flavus</i>	Aflatoxin	Mateles and Adye (1965)
2. <i>A. niger</i>	Malformin	Steenbergen and Weinbergen (1968)
3. <i>Claviceps paspali</i>	Lysergic acid	Rosazza <i>et al.</i> (1967)
4. <i>C. purpurea</i>	Ergotamine	Stoll <i>et al.</i> (1957)
5. <i>Fusarium vasinfectum</i>	Fusaric acid	Kalyansundaram and Saraswathi-Devi (1955)
6. <i>Penicillium chrysogenum</i>	Penicillin	Foster <i>et al.</i> (1943) Koffler <i>et al.</i> (1947)
7. <i>P. griseofulvum</i>	Griseofulvin	Grove (1967)
8. <i>P. usticae</i>	6-Methylsalicylate	Ehrensvarð (1955)
9. <i>P. usticae</i>	Gentisyl alcohol	Ehrensvarð (1955)
10. <i>P. usticae</i>	Patulin	Brack (1947)

ency influences diverse metabolic processes like carbohydrate metabolism including organic acid fermentation (Waksman and Foster, 1938; Foster and Waksman, 1939; Chesters and Robinson, 1951) and aerobic and anaerobic respiration (Grimm and Allen, 1954; Vallee and Hoch, 1955), amino acid metabolism (Nason *et al.*, 1951, 1953), ascorbic acid synthesis (Kalyanasundaram and Saraswathi Devi, 1955 b), nucleic acid metabolism (Nason *et al.*, 1951, 1953), production of certain antibiotics (Jarvis and Johnson, 1946, 1947; Kalayansundaram and Saraswathi Devi, 1955 a) etc. Zinc concentration of the medium influences the quantity of many primary and secondary metabolites; some of those reported in the recent past have been presented in Table 11.1.

### Copper

Requirement of copper by fungi as micronutrient was also recognised by classical researchers. Steinberg (1936, 1950) noted that highly purified media lacking copper supported reduced growth of a number of fungi, including *Aspergillus niger*, *Fusarium oxysporum*, *Cercospora nicotianae*, *Sclerotium rolfsii*, *Thielaviopsis basicola* and *Pythium irregulare*. Minute quantity or even small traces of copper has been found to be essential for a number of other fungi (Wolf and Emmerie, 1930; McHargne and Calfee, 1931; Starkey and Waksman, 1943; Nicholas, 1952; Tandon and Agarwala, 1956; Grewal, 1956; Agarwal, 1959; Tandon and Chandra, 1962). Sporulation of fungi is also affected by copper concentration of the medium (Steinberg, 1939), however, different fungi seem to differ in their response. Agarwal (1959) observed that *Fusarium coeruleum* strains exhibited stimulated sporulation in presence of Cu, but *Curvularia penniseti* could attain only poor sporulation. In a few fungi, however, essentiality of copper is difficult to demonstrate (Hawker, 1950). Saraswathi Devi (1958) could not establish the indispensability of copper for growth of some soil Fusaria studied by her. Blank and Talley (1941) even found that copper was toxic to *Phymatotrichum omnivorum*. Leonian and Lilly, (1940) found copper toxic to *Phycomyces blakesleeanus*. The most important aspect governing the toxic or stimulating influence of copper on fungal-growth is obviously its concentration. In contrast to its nutritive role in minute quantities, may be cited its fungicidal effect on the majority of fungi as a component of various copper fungicides, at higher concentrations. However, Starkey and Waksman (1943), working on copper-tolerant fungi found



that atleast two of them were able to thrive even in a saturated solution of copper-sulphate. It must, therefore, be noted that although the usual dose of copper supplied to the medium ranges from 0.01 to 0.1 ppm of Cu, one must be cautious regarding concentration of this metal while making nutritional investigation on fungi.

Effect of copper is also determined by other metal-ions present in the medium, *i.e.* upon ion-antagonism. Marsh (1945) concluded that antagonistic action of magnesium, calcium and potassium against copper was due to the impaired absorption and utilization of copper. Horsfall (1957) also suggested a similar mechanism, as has been described earlier in this chapter. Apparao (1959) observed that the toxic effects of copper was almost counteracted by Fe and Mn ions, whereas zinc appeared to enhance the toxicity of copper. Toxic effect of copper and its antagonism by iron was also noted by Koffler *et al.* (1947) in penicillin production by *Penicillium chrysogenum*.

A number of enzymes have been shown to require copper as their co-factor, *e.g.* tyrosinase and laccase. It also influences the biosynthesis of enzymes. Yoshimura (1939) reported that catalase production by *Aspergilli* was dependent upon the presence of various trace metals including copper. Medina and Nicholas (1957) demonstrated that biosynthesis of nitrite and hyponitrite reductases required copper. Studies on pectic enzyme system of *Fusarium vasinfectum* by Subramanian (1956) have indicated that copper in low concentration ( $10^{-6}$  M) stimulated both pectin methyl esterase (PME) as well as polygalacturonase (PG) activities.

Copper is also known to exert a striking influence on pigmentation of coloured spores. It has been noted in *Aspergillus niger* (Javillier, 1939; Mulder, 1939) as well as *Trichoderma viride* (Brian and Hemming, 1950). Spore colouration in *A. niger* has been found to change from yellow to brown and ultimately to black as the concentration of copper is increased.

### Manganese

Studies on fungal requirements of manganese have also been confined to a relatively small number of fungi. Yet, it is now believed that fungi in general need this metal as a minor element in their nutrition. The essentiality of manganese, which is generally required in minute traces (0.005-0.01 ppm) is, however, difficult to demonstrate in some fungi, particularly by using the classical methods.

Nicholas and Fielding (1951) as well as Nicholas (1952) could record only little change in the mycelial dry weight of *Aspergillus niger*, when manganese was completely omitted from the culture media. However, by employing specialized techniques, Donald *et al.* (1952) observed a 50% reduction in growth of the same fungal species, viz. *A. niger*. Sulochana and Lakshmanan (1968) recorded from 50 to 75% decrease in the mycelial yields of some strains of *A. niger* in Mn-deficient culture medium. Earlier, Steinberg (1939) had also reported quite apparent decrease in the growth of *A. niger* in Mn-deficient medium. Such varying results from studies on the same fungal species may either be ascribed to non-identical experimental conditions, impurity of chemicals and glass wares, faulty technique etc. Donald *et al.* (1952 a) have, however, suggested that different strains of *Aspergillus niger* exhibited different Mn-requirements. Many more factors are involved in masking the Mn-deficiency symptoms. Data obtained by Saraswathi Devi (1954, 1962), from her carefully controlled experiment, during which she employed the techniques of Donald *et al.* (1952 b), indicated that the fungus under study attained higher dry-weight on Mn-deficient media, as compared to that attained on a complete medium. However, Sulochana and Lakshmanan (1968) consider that such results may be due to the fact that the dry-weights were recorded on the 6th day when 'plus all' cultures had sporulated heavily and hence gave a lower mycelial dry-weight as compared to that obtained from Mn-deficient cultures which did not sporulate at all. Steinberg's (1936) data also show that if the concentration of trace elements is increased beyond the optimum requirement, it results in the lowering of the growth ratio (growth with element: growth without element). Difficulties in demonstrating the essentiality of manganese has also been ascribed to its requirement in very minute traces (Tandon and Chandra, 1962). It has been observed that Mn-deficiency symptoms become more and more pronounced as the inoculum quantity is reduced, which suggests that fungi are able to detect and utilize even very minute traces of manganese which is carried along with the inoculum.

Manganese is known to affect the cellular concentration as well as activity of various enzymes (Marsh, 1945; Hofmann *et al.* 1950). Medina and Nicholas (1957) were able to demonstrate that manganese was essential for the activity of hydroxylamine reductase enzyme of *Neurospora crassa*. Level of other enzymes, like nitrite reductase and hyponitrite reductase was also found to be lower in

the mycelium of *N. crassa* grown in Mn-deficient media. Earlier Yoshimura (1939) had shown that catalase production by *Aspergilli* depended on various trace metals including manganese. Subramanian (1956), however, observed that presence of manganese in the culture medium in concentrations ranging from 0.025 to 0.20 ppm caused inhibition of pectin methylesterase (PME) and polygalacturonase (PG) activities, while complete removal of Mn from the medium resulted in remarkable increase in the PME and PG activities of the mycelium of the wilt pathogen *Fusarium vasinfectum*. Manganese is also known to act as an ion-antagonist against copper and zinc toxicity, although with lesser efficiency than iron (Apparao, 1959; Sadasivan, 1967).

Role of manganese has been noted in the biosynthesis of some primary and secondary fungal metabolites also. Clark *et al.* (1965) noted that manganese at  $0.02 \times 10^{-5}$  M concentration inhibits citric acid synthesis by *Aspergillus niger*. For synthesis of malformin, a secondary metabolite of *A. niger*, however, manganese was required upto  $0.1 \times 10^{-5}$  M concentration, and proved inhibitory only at  $1.0 \times 10^{-5}$  M concentration (Steenbergen and Weinberg, 1968).

### **Molybdenum**

Essentiality of molybdenum for nitrogen metabolism was recognised quite early not only for fungi and bacteria (including those able to fix atmospheric nitrogen) but for higher plants as well (Bortels, 1930; 1936). Among fungi, molybdenum requirement of *Aspergillus niger* was first recorded by Steinberg (1936 b), who found that its requirement was more pronounced when grown on nitrate containing medium than when ammonium nitrogen source was used (Steinberg, 1937). This does not mean that the fungus does not require MO in the presence of ammonium nitrogen. In any case, this metal is needed in extremely minute amount ranging from 0.1 parts per billion (Mulder, 1948 b; Nicholas, 1952) to 10.0 ppb (Steinberg and Bowling, 1939). *A. niger* is able to detect even more negligible concentration, viz. 0.02 ppb of this element. Nicholas and Fielding (1950) recorded a 57 mg increase in the mycelial output of *A. niger*, when only 0.1  $\mu$ g of molybdenum was added to 50 ml of the purified medium. Such astonishing sensitivity of *A. niger* to detect and respond to infinitesimal quantity of molybdenum (besides some other trace elements) makes study on its microbial requirement extremely difficult. Although certain specific techniques of its removal (e.g.

sulphide-co-precipitation technique of Nicholas) have been found to be satisfactory (Donald *et al.*, 1952 a), yet attempts to devise a more comprehensive method may not go unrewarded.

Higher molybdenum requirement during nitrate utilization has now been decisively correlated with the essentiality of this metal for nitrate reductase enzyme. Nicholas *et al.* (1954) found that omission of added molybdenum caused a decline in the synthesis of nitrate reductase in *Neurospora crassa* and *Aspergillus niger*. Further, Nicholas and Nason (1954 a) demonstrated that the nitrate reductase enzymes of *N. crassa* was a metallo-flavoprotein containing molybdenum, molybdenum as a functional constituent of nitrate reductase is now well established (Nicholas *et al.*, 1954; Nicholas and Nason, 1954 a, b; Nicholas and Stevens, 1955; Nicholas, 1959 a, b). Nason (1956) has accumulated such evidences which show that molybdenum functions as an electron carrier in enzymatic reduction of nitrate in *Neurospora crassa*, *Escherichia coli* as well as soybean. Such a role of molybdenum has further been suggested by data obtained from studies with electron paramagnetic resonance studies (EPR) with the purified enzyme from *Neurospora* (Nicholas, 1965). It seems likely that two molybdenum atoms with valence states of +5 and +6 are required for two electron transfer to nitrate.

Role of molybdenum in growth and metabolism of fungi growing with a nitrogen source other than nitrate is, however obscure, although it is gradually being realised that this metal has some other roles also, possibly in the activity of some other enzymes or so. Subramanian (1956) recorded that this metal could enhance the pectin methylesterase (PME) and polygalacturonase (PG) activities in mycelia of *Fusarium vasinfectum*. Further investigations may, thus, help in enlarging the area of influence of this metal.

### Calcium

Essentiality of calcium in the nutrition of fungi was first demonstrated as early as 1922, when Young and Bennett (1922) observed that *Rhizoctonia solani* could not grow in the absence of calcium. However, different fungi appear to differ in their calcium requirements to the extent that *Aspergillus niger* and *Fusarium oxysporum* var. *nicotianae* do not require this element at all (Steinberg, 1948, 1950). However, later studies have shown its essentiality for fungi belonging to diverse taxa, and Cochrane (1958) has enumerated 19 species of fungi and Actinomycetes which have been reported to

respond to added calcium (0.5 to 20 ppm) by stimulated growth. However, it seems definite that for some fungi the essentiality of calcium cannot be demonstrated. In still other fungi like *Chaetomium* sp., this element does not seem to be required for growth, but it has been reported to stimulate perithecial production (Basu, 1951). All these indicate that more careful investigations pertaining to the role of calcium in fungal nutrition in general is very much called for. This is particularly so, because, calcium is often regarded to influence various organisms including fungi, through its non-nutritional role in ion-antagonism against certain toxic monovalent cations, like hydrogen, sodium, potassium etc. and its wide range of optima, extremely variable response of various fungi, and its replacement by strontium to an appreciable extent (Lindeberg, 1944; Steinberg, 1948; Ingraham and Emerson, 1954) do indicate such a possibility.

#### **Cobalt, Scandium, Vanadium and Gallium**

Fungal requirement of cobalt has been very much in doubt, although evidences indicating its essentiality have been presented quite often (Marston, 1952; Ballantine, 1953). In other living organisms, like bacteria, actinomycetes and animals, cobalt is known to be essential for the synthesis of compounds of the vitamin B<sub>12</sub> group (cobalmins). Cobalt forms a constituent part of the tetrapyrrole ring of this vitamin. However, due to lack of knowledge regarding the vitamin B<sub>12</sub> synthesizing ability of fungi, cobalt-essentiality for fungi also could not find adequate support. Now, fungi have been shown to synthesize cobalmins (Tanner, 1960) and in some cases natural deficiency for this vitamin has also been reported (Adair and Vishniac, 1958; Littman and Miwatani, 1963). Therefore, the earlier findings regarding essentiality of cobalt for fungi deserve reconsideration.

Essentiality of gallium, scandium and vanadium in fungal nutrition could not be concluded on the basis of a few scattered reports presently available. Most of the data were recorded in *Aspergillus niger* by Steinberg (1938, 1939, 1950) and Bertrand (1941, 1943), which shows that very little attention has been paid to their possible role in fungal physiology, particularly during recent times.

## VITAMINS AND GROWTH FACTOR IN FUNGAL NUTRITION

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Importance of vitamins in fungal nutrition was first recognised in the beginning of this century by Wildiers (1901), who designated these nutritional factors as "Bios". Subsequently, Pekelhasing, (1905) (cf. White *et al.*, 1959) indicated that certain food-stuffs contained an unknown substance, which even in very small quantity was of paramount importance to nourishment. A few years later *i.e.* in 1912, Hopkins and Funk proposed their vitamin theory, according to which absence of a particular nutritional principle from animal diet resulted in various kinds of disease. The first essential food-factor isolated by Funk was an amine, which led him to designate that compound as "vitamine" *i.e.* an amine, necessary for life. The term has now acquired a wide application and represents a class of compounds, which are considered distinct from the major components of food like carbohydrates, lipids, amino acids, minerals and water. The vitamins are effective in minute quantities and are necessary or stimulatory for growth. Generally, compounds used as energy sources or as structural components are not included under vitamins. Vitamins in minute quantities are effective in many of the biochemical reactions of the cell, which obviously reflects their role as catalysts. It is now known that most of the vitamins perform their catalytic role as coenzymes or constituent parts of coenzymes (Table 12.1). It is perhaps on this account that vitamins are essential for diverse groups of organisms ranging from microbes to man.

Fungi, in their ability to synthesize their vitamin requirement occupy a position in between the totally independent higher plants and completely dependent animals. They exhibit all the possible transitional stages in their capacity for vitamin synthesis. Some of the fungi



TABLE 12.1

SHOWING SOME WATER-SOLUBLE VITAMINS, THE COENZYMES DERIVED FROM THEM, AND THE REACTIONS WHICH THEY CATALYZE

<i>Vitamin</i>	<i>Co-enzyme</i>	<i>Reactions involved</i>
Vitamin B <sub>1</sub> (Thiamine)	Thiamine pyrophosphate	Decarboxylation of $\alpha$ -keto acids; certain reactions of keto sugars.
Vitamin B <sub>2</sub> (Riboflavin)	Flavin mononucleotide Flavin adenine dinucleotide	Various oxidation reduction reactions.
Vitamin B <sub>6</sub> (Pyridoxin)	Pyridoxal phosphate	Reaction involving amino acids, like decarboxylation, transamination.
Nicotinic acid (niacin)	Nicotinamide adenine dinucleotide (NAD <sup>+</sup> ) Nicotinamide adenine dinucleotide phosphate (NADP <sup>+</sup> )	} Various oxidation reduction reactions.
Pantothenic acid	Coenzyme A (CoA-SH)	
Biotin	Enzyme-bound biotin	Reactions involving CO <sub>2</sub> fixation, fatty acid synthesis, carboxylation etc.
Folic acid	Tetrahydrofolic acid	Reactions involving one-carbon compounds.
Vitamin B <sub>12</sub> (Cyanocobalamin)	"Cobamide" coenzyme	Carbon-chain isomerizations, methyl group transfers.

are capable of meeting all of their vitamin-requirements by themselves, others may be partially deficient, and still others may be completely deficient for one or more vitamins. Schopfer (1943) has distinguished two groups of fungi, viz., (i) the auxoautotrophic and (ii) auxoheterotrophic. The auxoautotrophic fungi are able to synthe-

size all their vitamin-requirement and some of them may even synthesize a vitamin, far in excess of their own requirement, which may be excreted into the medium. Such fungi are exploited for commercial production of vitamins, e.g. *Ermothecium ashbyii* and *Ashbya gossypii* are employed industrially for the production of riboflavin (vitamin B<sub>2</sub>). The auxoheterotrophic group, includes those fungi which either completely or partially lack biosynthetic capacity for vitamin production. Fungi which are partially deficient in vitamin synthesis make limited growth on a vitamin free medium and show a marked stimulation when provided with an exogenous source of vitamin. The partial vitamin deficiency is not permanent and may be overcome by changing the external conditions of growth, but total vitamin-deficiency is always absolute and unalterable.

Although, this field of fungal nutrition got its real fillip only after Schopfer (1934 b) demonstrated the role of thiamine on *Phycomyces blakesleeana*, much ground has been covered during the last 20 years or so. The topic has been reviewed well by several authors, including Robbins and Kavanagh (1942), Schopfer (1943), Knight (1945), Lilly and Barnett (1951), Cochrane (1958) and Fries (1961, 1965). In many of the recent researches on this aspect, artificially induced mutants have been employed, and it is now well recognised that biosynthesis of each growth-factor is regulated by numerous genes. Mutation in any one of them may obviously interfere with the biosynthetic pathway and thus block the production of a particular vitamin. Work with induced mutants has been carried on with *Neurospora*, *Ophiostoma*, *Aspergillus* and several other fungi (Fincham and Day, 1963). Such mutations may also arise spontaneously in the natural population although to a limited extent. This possibly explains the observation that the capacity to synthesize a particular vitamin may change with time in the same strain. It has also been suggested that different degrees of vitamin-deficiency in fungi also operate through a complicated genetic mechanism (Lilly and Barnett, 1948 a). In fact the accumulated genetical data have led to a concept that auxoheterotrophic strains have originated from auxoautotrophic stock, chiefly by spontaneous mutations (Lwoff, 1943; Schopfer, 1944 b).

### VITAMIN REQUIREMENTS OF FUNGI

Our current knowledge of vitamin requirements of fungi indicates that they generally need only water-soluble vitamins of B-complex series, including thiamine (B<sub>1</sub>), riboflavin (B<sub>2</sub>), pyridoxin (B<sub>6</sub>), niacin



(nicotinic acid), pantothenic acid, biotin (H), folic acid group, inositol, *p*-aminobenzoic acid and cyanocobalmin ( $B_{12}$ ). None of the fat-soluble vitamins like A, D, E and K have so far been found to be synthesized by fungi, and it appears that they do not require these growth-factors. However, a number of growth-factor requirements of fungi are still poorly understood and therefore any extreme and hasty conclusion in this regard needs caution. Moreover, several fungi are known to respond with stimulated growth to various natural materials, which may contain unknown growth factors, because in many such cases identical growth response could not be induced by addition of specific purified vitamins or other nutrients.

### Thiamine (Vitamin $B_1$ )

**Structure.** Thiamine molecule consists of two moieties, *viz.* (i) 2, 5-dimethyl 6-amino pyrimidine (simply referred as pyrimidine), and (ii) 4-methyl-5-hydroxyethyl thiazole commonly called as thiazole. These two components can be chemically or biologically made to couple leading to the synthesis of thiamine. Its chemical structure as well as those of its two components are represented below.

Information on the synthesis, occurrence as well as history of this vitamin are available from Williams and Stries (1938), Rosenberg (1942), and Schopfer (1943).

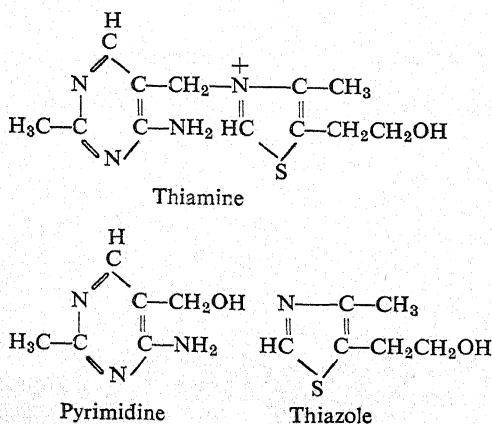


Fig. 12.1. Structure of thiamine, pyrimidine and thiazole.

**Metabolic role.** In the form of thiamine pyrophosphate (TPP), this vitamin is long known to perform the functions of coenzyme catalyzing the decarboxylation of  $\alpha$ -keto acids, like pyruvic acid,  $\alpha$ -keto-glutaric

acid etc. Role of TPP in the pyruvate decarboxylation in fungi is evident from accumulation of pyruvate in thiamine deficient cultures (Haag, 1940; Wirth and Nord, 1942; Friend and Goodwin, 1954) as well as enhanced ethanol production in its presence (Dammann *et al.*, 1938; Schopfer and Guilloud, 1945). TPP is also a coenzyme in transketolation reactions of pentose-phosphate pathway (Jensen, 1954) and helps in the transfer of the glycolaldehyde moiety to the aldose. Thiamine has also been reported to promote cytochrome synthesis in *Ustilago sphaerogena* (Grimm and Allen, 1954) and prevents oxalate accumulation (Nagate *et al.* 1954).

*Fungal requirements.* Thiamine is required by the largest number of fungi, and possibly on this account, it was the first vitamin to be demonstrated as essential for *Phycomyces blakesleanus* (Schopfer, 1934; Burgeff, 1934). Subsequent studies on thiamine-requirement of fungi have shown that majority of fungi belonging to diverse taxa are auxoheterotrophic for this vitamin. Among Phycomycetes, the number of thiamine-deficient species is not very large, but considerable. Most of the species of *Phytophthora* (Robbins, 1938) and *Phycomyces* (Leonian and Lilly, 1938; Robbins, 1938 b; Robbins and Kavanagh, 1938 a) as well as *Mucor ramannianus* (Muller and Schopfer, 1937; Muller, 1941), *Allomyces kniepii* (Quantz, 1943), *Blakeslea trispora* (Leonian and Lilly, 1938) and *Blastocladiella emersonii* (Barner and Cantino, 1952) require thiamine. On the contrary, species of *Mortierella* (Robbins and Kavanagh, 1938 b) and *Rhizopus* (Schopfer, 1935) are completely auxoautotrophic barring a few exceptions only.

Thiamine-requirement of Ascomycetes has not been investigated very much and has mostly been confined to yeasts. Available reports suggest that yeasts as well as some other, Ascomycetes show multiple vitamin requirements including that of thiamine, e.g. *Saccharomyces*, *Kloeckera brevis*, *Zygosaccharomyces japonicus* (Burkholder *et al.* 1944), *Rhodotorula aurantiaca* (Robbins and Ma 1944), *Ermothecium ashbyii* (Schopfer and Guilloud, 1945), *Chaetomium* (Lilly and Barnett 1949), *Trichophyton* (Robbins and Ma, 1945), *Glomerella* (Srinivasan and Vijayalakshimi, 1960), *Sordaria* (Fields and Maniotis, 1963), etc. Thiamine heterotrophy is most common among Basidiomycetes. Most of the species investigated under the following genera were found to be thiamine-deficient; *Boletus* (Melin and Nyman, 1940, 1941; Melin and Norkrans, 1942), *Clitocybe* (Lindeberg, 1946 a), *Coprinus* (L. Fries, 1945, 1955), *Exobasidium* (Sundstrom, 1960), *Marasmius*

(Lindeberg, 1944), *Mycena* (Fries, 1949), *Peniophora* (Fries, 1950), *Polyporus* (Fries 1938; Noecker, 1938), *Tricholoma* (Norkrans, 1950), and *Lactarius* (Jayko *et al.*, 1962) *etc.* Sadasivan and Subramanian (1954) have listed several fungi, which are either partially or totally deficient for thiamine. Many of the imperfect fungi have also been reported to be thiamine requiring. Some important ones include the species of *Phyllosticta* (Bilgrami, 1963; Tandon, 1967), *Gloeosporium* spp., *Colletotrichum papayae* and *Pestalotia mangiferae* (Tandon, 1967), *Pestalotia pauciseta* and *Botryodiplodia theobromae* (Prasad, 1966), *Sclerotium rolfsii* (Sahni, 1967), some strains of *Colletotrichum gloeosporioides* (Singh and Prasad, 1967) *Helminthosporium solani* (Singh, 1973) and *Cercospora cruenta* (Jandaik and Kapoor, 1972). It has been observed that different fungal species differ in their mode of thiamine requirements. While a few fungi require the intact thiamine molecule for their optimum growth, majority of them can do equally well or even better (Norkrans, 1950) when the two components of this vitamin are supplied separately in equimolar concentrations. Many fungi are even capable of doing away with one or the other component of the vitamin, which obviously indicates that such organisms have not only the capacity to synthesize the other moiety of this vitamin but they are also able to bring about a coupling of the two moieties and synthesize thiamine, because none of the moieties is individually active as vitamin. Available reports (Robbins and Kavanagh, 1942, 1944; Cochrane, 1958) suggest that ability to synthesize pyrimidine moiety is less common which is indicated by the requirement of pyrimidine by a large number of fungi. Thiazole, on the other hand, is required by only a few fungi, which shows that many of the fungi are capable to synthesize this component of the vitamin. Biosynthesis of thiamine has often been supposed to be a simple and direct condensation phenomenon of its two components, *viz.* pyrimidine and thiazole. However, evidences though indirect, have been adduced suggesting an indirect pathway of its biosynthesis (Harris, 1956), which may be schematized as below:

Pyrimidine + thiazole precursor → thiamine-like intermediate → thiamine

In contrast to its general role as a growth promoting factor, certain fungal species respond to thiamine with growth-inhibition or they destroy or inactivate atleast a part of this vitamin. Inhibition of growth due to addition of thiamine has generally been recorded in fungi auxoautotrophic for this vitamin, including species of *Ciborinia* (Lilly and Barnett, 1948 b), *Fusarium* (Wirth and Nord, 1942; Elliott,

1949; Esposito *et al.*, 1962, Mathur *et al.*, 1964) and *Rhizopus* (Schopfer, 1935; Robbins and Kavanagh, 1938), *Colletotrichum lindemuthianum* (Mathur *et al.*, 1950). Such response has, however, been suggested (Schopfer and Guilloud, 1945) to be an effect of accelerated production and accumulation of ethyl alcohol due to increased availability of thiamine, because thiamine pyrophosphate (cocarboxylase) is the coenzyme in pyruvate decarboxylation. Inactivation or destruction of this vitamin has been recorded in two different fungi *viz.* *Phycomyces blakesleeanus* and *Sclerotium rolfsii*. Both these fungi are heterotrophic for thiamine and are able to utilize both intact thiamine as well as its two moieties. Irrespective of the form in which this vitamin is supplied to these fungi, a part of thiazole is either destroyed or inactivated through the activity of an enzyme. The temperature-relation of this enzyme may possibly explain the observation that thiamine is more active as a growth regulator at lower temperatures (Robbins and Kavanagh, 1944).

### Riboflavin

**Structure.** Riboflavin (Vitamin B<sub>2</sub>, vitamin G, lactoflavin) has the empirical formula C<sub>17</sub>H<sub>20</sub>N<sub>4</sub>O<sub>6</sub> and chemical name, 6, 7-dimethyl-9-(1-D-ribityl)-isoalloxazine. Structurally, it may be represented as follows:

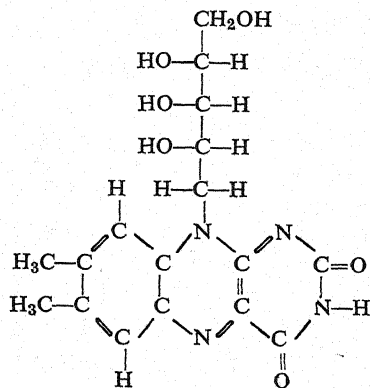
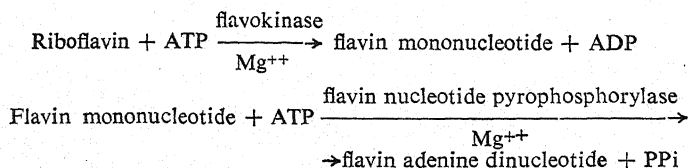


Fig. 12.2. Structure of riboflavin.

Riboflavin was first identified in 1935 by Kuhn and Karrer as a prosthetic group of an enzyme isolated from yeast by Warburg and

Christian in the year 1932. This enzyme could oxidize NADPH and had riboflavin 5'-phosphate (flavin mononucleotide, PMN) as its prosthetic group. Subsequently yet another riboflavin derivative, viz. flavin adenine dinucleotide (FAD) was found to act as the co-enzyme. Many riboflavin containing enzymes are now known. Both these active forms of riboflavin, viz. PMN and FAD are produced by phosphorylation reactions with ATP under the influence of specific enzymes, as shown below:



*Metabolic role.* Riboflavin is now known to comprise the prosthetic group of a multitude of oxidizing enzymes, known collectively as flavin enzymes and thus plays a fundamental role in metabolism. The flavo-proteins (flavin containing enzymes) perform the important function of reoxidizing the reduced NADH or NADPH, and thus ensure the cell, an uninterrupted availability of these coenzymes in oxidized form ( $\text{NAD}^+$  and  $\text{NADP}^+$ ), which in turn are essential for the functioning of the dehydrogenases they belong to. The FMN or FAD, which get reduced in the process, are reoxidized by one of the cytochrome enzymes, which are heme-proteins. Some of the flavoprotein dehydrogenases are, however, unable to negotiate directly with the cytochrome chain, and a specific enzyme, viz. electron transferring flavo-protein, mediates in such cases by accepting and donating electrons from the former the latter. A few flavoproteins may even be reoxidized directly by  $\text{O}_2$  and are autoxidizable. These enzymes are designated as aerobic dehydrogenases. *Penicillium notatum* and *P. resticulosum* are known to produce a glucose oxidase, which is an aerobic dehydrogenase. The D- and L-amino acid oxidases are also flavin containing aerobic dehydrogenases.

Riboflavin derivatives constitute the prosthetic groups of several other enzymes also, which include nonautoxidizable dehydrogenases like succinic dehydrogenase, and the cytochrome-linked lactic dehydrogenase. Some of the flavoproteins are also metalloproteins, containing molybdenum, iron, copper etc. A molybdoflavoprotein catalyzes the reduction of nitrate, whereas a copper containing flavoprotein acts as nitrite reductase.

No other role is known for riboflavin either in fungi or any other biological system, except for some indirect effect on synthesis of compounds like carotenoids (Zalokar, 1954, 1955), nicotinic acid (Dalglish, 1955) etc.

**Fungal requirements.** Fungi appear to be more or less autoauxotrophic for riboflavin, as there is upto now only a singular report of riboflavin-heterotrophy among fungi. Jennison *et al.* (1955) reported that *Poria vaillantii* requires an external source of riboflavin. Otherwise riboflavin is known to be synthesized by many yeasts and other related species, and a large number of filamentous fungi. *Ashbya gossypii*, *Ermothecium ashbyii* and *Candida* spp. are prolific producers of riboflavin and are commercially harnessed for this vitamin. However, some riboflavin requiring mutants of *Neurospora* and *Aspergillus* have been isolated, and requirements for this vitamin has been shown in slime molds, *viz.* *Dictyostelium* spp., (Sussman, 1956) and several bacterial species, particularly lactobacilli. This has aroused fresh interest in the fungal requirements of this vitamin which need further attention.

### Pyridoxine

**Structure.** Pyridoxine was isolated from liver cells in the year 1938 and was synthesized a year later in 1939. Subsequently it was observed that two of its closely allied derivatives *viz.* pyridoxal and pyridoxamine were also or even more active as vitamin. All these three compounds are together referred to as vitamin B<sub>6</sub>, as they differ only slightly in their structure, *i.e.*, in the presence of either a primary alcohol or an aldehyde or a primary amine group in their molecule, as shown below:

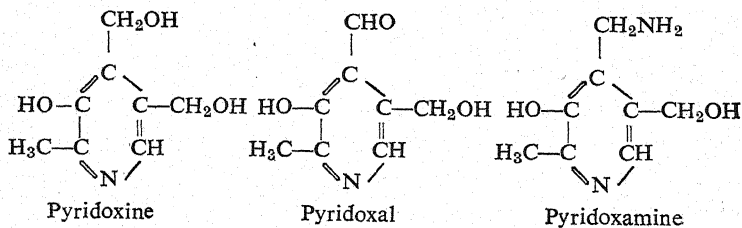


Fig 12.3. Structure of pyridoxine, pyridoxal and pyridoxamine.

*Metabolic role.* Many metabolic transformations of amino acids, like decarboxylation, transamination, synthesis of tryptophan etc. require pyridoxal phosphate as the coenzyme. It is, therefore, suggested that pyridoxine might not be acting directly as vitamin, rather it might be functioning as a precursor of pyridoxal, which after phosphorylation by ATP, yields the coenzyme pyridoxal phosphate.

In *Neurospora crassa* pyridoxal phosphate has been shown to participate in a variety of enzymic reactions (Yanofsky, 1932; Umbreit *et al.*, 1947; Strauss, 1951; Reissing, 1952) including reduction of nitrite (Silver and McElroy, 1954). Evidences obtained from animal cells indicate that pyridoxal or its phosphate ester also plays a fundamental role in active transport of amino acids and metal ions across cell-membranes, serving as carrier.

*Fungal requirements.* Several fungi belonging to Ascomycetes and Fungi Imperfecti have been reported to require this vitamin as a growth-factor but the list of such organisms is far more concise than that for thiamine. Pyridoxine requirement by fungi was first demonstrated for *Saccharomyces cerevisiae* (Schultz *et al.* 1938) and was soon extended to several species of yeast (Schultz *et al.*, 1939; Eakin and Williams, 1939; Burkholder, 1943; Snell and Rannefeld, 1945). Among the filamentous fungi pyridoxine-heterotrophy has been reported in *Ophiostoma* spp. (Fries, 1942, 1943; Robbins and Ma, 1942 b, c), *Trichophyton discoides* (Robbins *et al.*, 1942), *Ascoidea rubescens* (Fries, 1943), *Ceratocystis pilifera* (Leaphart, 1956), *Leptographium* spp. (Leaphart, 1956), *Colletotrichum capsici* (Misra and Mahmood, 1961) and *C. gloeosporioides* (Prasad, 1966) etc.

The lone report of a pyridoxine requiring Basidiomycete concerns *Ustilago maydis* which utilizes vitamin B<sub>6</sub> and exhibits enhanced synthesis of indoleacetic acid from tryptophane (Alighisi *et al.*, 1964). The three constituents of vitamin B<sub>6</sub> viz. pyridoxine, pyridoxal and pyridoxamine appear to be of almost similar value to fungi (Snell and Rannefeld, 1945; Melnick *et al.*, 1945) although further investigations on this aspect may be more revealing, particularly because some of the bacteria utilize them differently. Also *Saccharomyces cerevisiae* attains best growth on pyridoxine and some pyridoxine-specific mutants of *Ophiostoma multiannulatum* have been reported (Wikberg, 1959). Pyridoxineless mutants of *Neurospora crassa* and *N. sitophila* have also been obtained, but their requirement for this vitamin is reported to be conditioned by various factors like presence or absence

of thiamine (Stokes *et al.*, 1943; Tatum and Bell, 1946), pH of the media (Strauss, 1951), etc.

### Nicotinic Acid (Niacin)

**Structure.** Nicotinic acid, an oxidation product of nicotine has long been identified as a part of the phosphopyridine coenzymes NAD and NADP. In fact, its metabolic role through these coenzymes was anticipated well before its nutritional significance was authentically established. It is believed that the active form of nicotinic acid is nicotinamide, although different organisms exhibit varying capacity to transform nicotinic acid into its amide, and also the enzyme catalyzing such transformation has not yet been isolated. The structures of nicotinic acid and its amide are shown below:

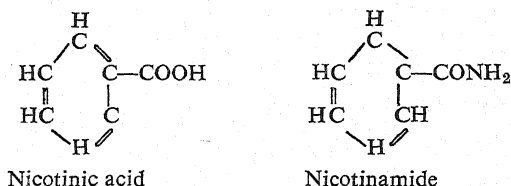


Fig. 12.4. Structure of nicotinic acid and nicotinamide.

**Metabolic role.** As component of NAD and NADP (the coenzymes of various dehydrogenases) the nicotinamide, which is the active biological derivative of this vitamin, participates in essentially all the oxidation reduction reactions occurring within living cells. Also it is due to the nicotinamide, that the coenzymes NAD and NADP are capable of being reversibly oxidized and reduced (Fig. 12.1) and thereby serve as oxidizing and/or reducing agents.

No other metabolic role has been assigned to this vitamin.

**Fungal requirement.** Niacin-heterotrophy has been frequently reported both in yeasts and filamentous fungi, and the deficiency appears to be more common among, the former. A number of yeasts, including *Torula*, *Mycotorula*, *Candida Kloeckera* as well as *Saccharomyces* have been reported to be niacin-deficient (Burkholder, 1943; Burkholder *et al.*, 1944; Wright, 1943; Miyashita *et al.*, 1958). Rogosa (1943) found that all the 114 strains of yeast that he studied, were niacin-deficient. Leonian and Lilly, (1942) reported that *Saccharomyces cerevisiae* exhibited strain differences with regard to their requirement



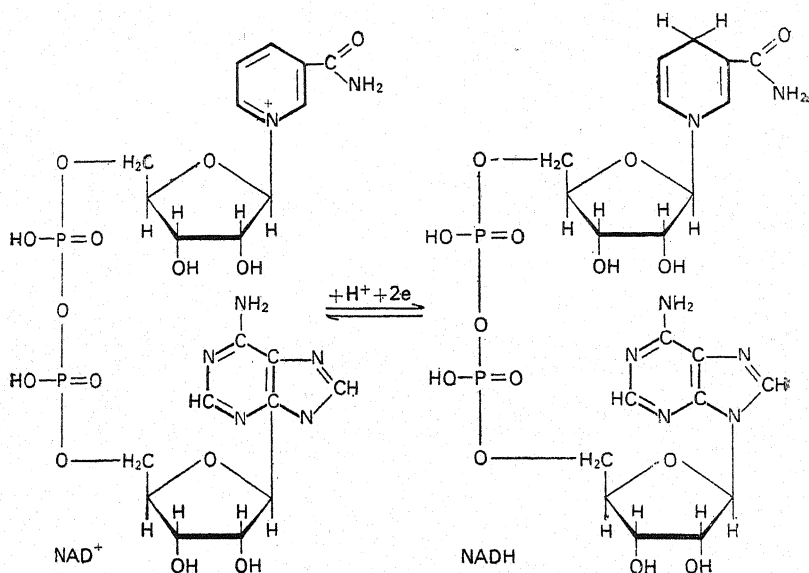


Fig. 12.5. Role of nicotinamide in oxidizing reducing activities of NAD/NADP.

for this vitamin. The fact that the filamentous fungi were niacin-deficient was discovered rather late. Cantino (1948) reported *Blastocladiella pringsheimii* as completely deficient for niacin. Since then some more phycomycetous fungi, including *Blastocladiella ramosa* (Crasemann, 1957), *Phlyctorhiza variabilis* (Rothwell, 1956) etc. have been added to the list. Some other filamentous fungi reported to require this vitamin either belong to Ascomycetes, e.g. *Venturia inaequalis*, (Fothergill and Ashcroft, 1955), *Trichophyton equinum* (Georg, 1949 a), *Glomerella cingulata* (Struble and Keitt, 1950); or to imperfect fungi, e.g., *Microsporum audouinii* (Area Leao, and Cury, 1950). In Basidiomycetes, however, niacin-deficient fungi are yet to be recorded, although *Pholiota aurea* is able to grow with niacin as the only growth-factor (Bach, 1956) and niacin-less mutants may be isolated from niacin-independent population of *Polyporus abietinus*.

Niacin-less mutants are rather easy to induce, and in fact induced or spontaneous mutants for this trait have been isolated in various fungi, including *Ophiostoma multiannulatum* (Fries, 1948), *Glomerella cingulata* (Andes and Keitt, 1950), *Neurospora crassa* and *Aspergillus*

*niger* the last two being very much helpful in studies relating to the pathway for niacin-biosynthesis.

### Pantothenic Acid

**Structure.** Like thiamine, pantothenic acid molecule is also composed of two different moieties, viz.,  $\beta$ -alanine and pantoic acid, which are held together by an amide linkage. This compound was first recognised as a growth factor in the yeast, *Saccharomyces cerevisiae* (Williams *et al.* 1932), but soon the ubiquitous occurrence of this vitamin was realised and the factor was accordingly named as pantothenic acid (Gr. pantos, everywhere). In 1940, pantothenic acid was isolated and its chemical structure was elucidated.

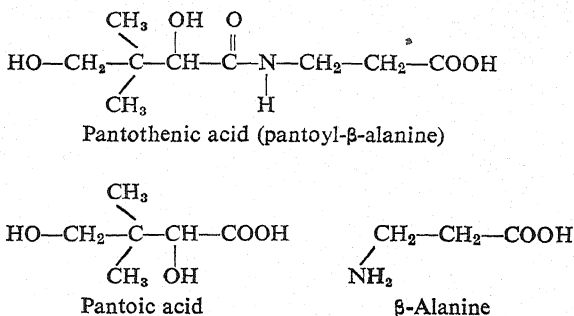


Fig. 12.6. Structure of pantothenic acid and pantoic acid.

**Metabolic role.** Pantothenic acid is known to form a major fraction of the coenzyme A, which catalyzes all the biological reactions concerning transfer of acetyl group. In fact the symbol 'A' given to this coenzyme indicates the importance of this coenzyme in acetylation reactions. However, it is now known that the role of coenzyme 'A' is not limited to acetyl transfer only, rather it helps in the fatty-acid metabolism in general.

**Fungal requirements.** Pantothenic acid heterotrophy seems to be more frequent among the yeasts than in the filamentous fungi. A number of species of genera like *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Candida* and *Torula* have been reported to be partially or completely deficient for this vitamin. As in case of thiamine, the deficiency for pantothenic acid also, may either be for the intact vitamin molecule or for one or both moieties. Some of the

yeasts, like *Saccharomyces cerevisiae* (Weinstock *et al.*, 1939), *Schizosaccharomyces pombe* (McVeigh and Bracken, 1955) etc. are able to thrive well in the presence of the  $\beta$ -alanine moiety only, and hence it is suggested that they synthesize the other component pantoic acid and bring about their coupling. Capacity to synthesize  $\beta$ -alanine, however, appears to be less common.

Among the filamentous fungi, the only fungus which exhibits almost an absolute deficiency for this vitamin is the basidiomycete *Polyporus texanus* (Yusef, 1953). In fact, this fungus requires only the pantoic acid moiety, and in contrast to most of the yeasts, seems to be capable of synthesizing  $\beta$ -alanine. *Penicillium digitatum* seems to exhibit a partial heterotrophy for this vitamin (Wooster and Cheldelin, 1945), as pantothenic acid has been reported to stimulate this fungus in liquid culture (Fergus, 1952). Recently, Satyavir and Grewal (1973) have reported stimulated growth of *Fusarium coeruleum* in presence of this vitamin. Such rare occurrences of pantothenic acid deficiency among filamentous fungi do not exhibit a limited significance of this vitamin for these organisms. Rather, this possibly reflects that auxoautotrophy for this vitamin is the rule among filamentous fungi. Evidences to support such a generalisation have been obtained but from a few fungi, including *Boletus edulis* (Dagys and Bluzmanas, 1943), *Neurospora crassa* (Wagner and Hoddock, 1951) and others (Nielsen and Hartelius, 1945). Further work on this line is likely to be rewarding and revealing.

### **Biotin**

*Structure.* Biotin also belongs to the B-complex family of vitamins. Isolated from egg-yolk, Kogl and Tonnies (1936) recognised its growth promoting activities and named it as biotin. Earlier it was designated as coenzyme R, because it was found necessary for growth and respiration of the bacterium *Rhizobium*. Williams *et al.* (1940) reported it as an essential growth factor for certain yeasts. Structure of this vitamin was established by Du Vigneaud *et al.*, (1942 a), and its synthesis was achieved by Harris *et al.* (1943). Biotin molecule comprises a single tetrahydrothiophene ring with a side chain of four methyl groups. Although in some related compounds the number of methyl groups may be less (norbiotin with 3) or more than four (homobiotin with 5). Some other biotin-analogues showing minor differences from natural biotin have also been studied. Structure of biotin and two of its allies are shown on the next page:

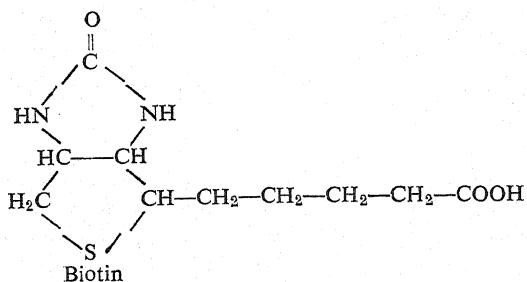


Fig. 12.7 a. Structure of biotin.

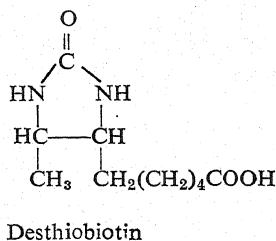
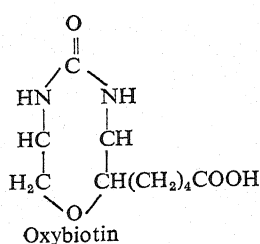
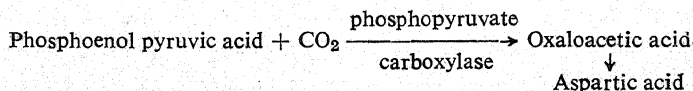


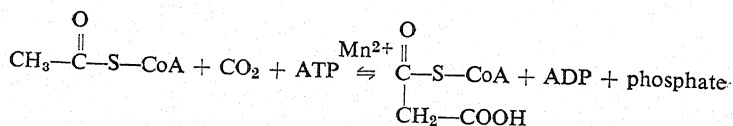
Fig. 12.7 b. Structure of oxybiotin and desthiobiotin.

It may be noted that in oxybiotin the sulphur of the biotin molecule has been replaced by oxygen, and in desthiobiotin sulphur has been removed altogether leading to a change in the tetrahydrothiophene-ring.

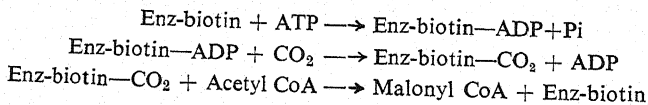
*Metabolic role.* Data obtained from studies with biotin indicate that this vitamin has some definite role to perform in a variety of cellular processes in fungi, although much remains to be understood regarding the manner in which biotin participates in these reactions. Generally, this vitamin has been associated with reactions involving fixation of  $\text{CO}_2$  into large organic molecules. Gyorgy (1954) suggested that biotin controls the synthesis of aspartic acid either during carboxylation of pyruvic acid or during conversion of oxaloacetic acid to aspartate:



Another carboxylation reaction influenced by biotin and reported to occur in fungi (Bu'lock and Smalley 1961) is the conversion of acetyl CoA into malonyl CoA.



Role of biotin in this reaction has been studied in greater detail and it has been suggested that biotin acts as the prosthetic group of the acetyl CoA carboxylase enzyme. In this position biotin has the capacity to react with  $\text{CO}_2$  to form its N-carboxyl derivative, provided that the biotin-enzyme complex has been pre-activated by ATP.



Synthesis of malonyl CoA from acetyl CoA seems to be quite common among fungi because malonyl CoA is known to participate in the synthesis of fatty acids, polyacetylenes, polyketide (Bu'lock and Smalley, 1961) and penicillic acid (Bentley and Keil, 1961). Bu'lock and Smalley (1961) during their experiment with labelled ethyl malonate found that when malonate was not supplied, the fungus synthesized it from acetyl CoA and carbon dioxide. A similar synthesis of malonate cannot be denied in other synthetic reactions where malonate is required. However, the role of biotin in malonate synthesis in fungi is yet to be fully assessed, although in fatty-acid synthesis biotin has been reported to exert its influence (Hodson, 1949; Mathiesen, 1950; Fries, 1965) which may be acting through malonate synthesis.

Other cellular activities of fungi influenced by biotin in one way or the other, include phosphorylation reactions catalyzed by hexokinases, deamination of some amino acids, ornithine cycle as well as utilization of ammonia (Fries, 1943).

*Fungal requirements.* So far, biotin-requirement among fungi appears to be next only to thiamine, and is more common among the yeasts than the filamentous forms. However, auxoheterotrophic species belonging to all the major taxonomic groups of mycelial forms are already on record.

It has been observed that biotin-requiring fungi exhibit some characteristic response to other growth factors. Generally, biotin deficiency is accompanied by a thiamine-heterotrophy. Those exhibiting such behaviour include *Helminthosporium solani* (Singh, 1973), *Gloeosporium musarum*, *G. papayae* and *Colletotrichum papayae* (Tandon, 1967), *Pestalotia pauciseta* (Prasad, 1966), *Colletotrichum gloeosporioides* (Singh and Prasad, 1967), *Phyllosticta bauhiniae*, *P. caricapapayae* and *P. pandanicola* (Bilgrami, 1963), and species of *Sordaria* (Fields and Maniotis, 1963), *Lactarius* (Jayko *et al.*, 1962), *Gloeocercospora* (Malca and Ullstrup, 1960), *Glomerella* (Srinivasan and Vijayalakshmi, 1960), *Isaria* (Taber and Vining, 1959), *Sepedonium* (Paninter, 1954), *Pyricularia* (Sadasivan and Subramanian, 1954; Leaver *et al.*, 1947), *Podospora* and *Endothia* (Lilly and Barnett, 1951), *Trichosporon* (Area Leao and Cury, 1950), *Lachnum* and *Spathularia* (Fries, 1950), *Chaetomium* (Lilly and Barnett, 1949), *Collybia* (Lindeberg, 1946 b), *Saccharomyces* (Burkholder *et al.*, 1944), *Ophiostoma* (Fries, 1943; Robbins and Ma, 1942 b), *Debaryomyces* (Burkholder, 1943), *Ophiobolus* (White, 1941), *Marasmius* (Lindeberg, 1939) as well as *Melanconium*, *Hypoxydon*, *Lophodermium* and *Valsa* (Fries, 1938). Fungi like *Lambertella pruni*, *Coemansia interrupta* and *Pleurogace curvicolli* also exhibit heterotrophy for biotin and thiamine simultaneously.

In other fungi, biotin-heterotrophy has either been found to be tagged with deficiency for some vitamin other than thiamine, or with none at all. The group showing the former response includes besides many yeasts, fungi like *Ascoidea rubescens* (Fries, 1943), *Kloeckera brevis* (Burkholder *et al.*, 1944), *Poria vaillantii* (Jennison *et al.*, 1955), *Fusarium oxysporum* f. *cumini* (Mathur *et al.*, 1964), some strains of *Colletotrichum gloeosporioides* (Singh and Prasad, 1967) and *Fusarium caeruleum* (Satyavir and Grewal, 1973). Several fungi, like *Penicillium digitatum* (Wooster and Cheldelin, 1945), *Sclerotinia camelliae* (Barnett and Lilly, 1948) and *Cercospora cruenta* (Jandaik and Kapoor, 1972) exhibit multiple vitamin deficiency including that for biotin. On the contrary, auxoheterotrophy, exclusively for biotin has also been observed in many fungi, including *Melanospora destruens* (Hawker, 1934), as well as species of *Dipodascus* (Batra, 1963), *Gloeosporium*, *Nigrospora*, *Verticillium* (Esposito *et al.*, 1962), *Gelasinospora* (Hackbarth and Collings, 1961), *Sardaria* (Lilly and Barnett, 1947; Olive and Fantini, 1961), *Claviceps* (Taber and Vining, 1957), *Blastomyces* (Halliday & McCoy, 1955), *Allescheria* (Area Leao and

Cury, 1950), *Histoplasma* (Savin, 1949), *Memnoniella*, *Stachybotrys* (Perlman, 1948), *Debaryomyces*, *Hansenula*, *Torula* and *Torulopsis* (Burkholder, 1943; Burkholder and Moyer, 1944), *Candida* (Burkholder and Moyer, 1944; Miyashita *et al.*, 1958), *Mitrula* (Fries, 1943), *Grossmannia* (Robbins and Ma, 1942 a) and *Neurospora* (Butler *et al.*, 1941).

Utilization of biotin-derivatives by fungi have also been studied, which have largely contributed to the understanding of biosynthetic pathway for biotin. The complete biosynthetic pathway is however yet to be elucidated. Desthiobiotin and its utilization by some biotinless mutants of *Penicillium chrysogenum* led Tatum (1945) to suggest that desthiobiotin is a precursor of biotin, and pimelic acid that of desthiobiotin.

Pimelic acid  $\rightarrow$  Desthiobiotin  $\rightarrow$  Biotin

However, biotin occurs naturally as biocytin ( $\epsilon$ -N-Biotinyl-L-lysine) in many biological materials, which has also been reported to be equally active for various fungi, including *Neurospora crassa* and a biotinless mutant of *Penicillium chrysogenum* (Wright *et al.*, 1952), *Isaria cretacea* (Taber and Vining, 1959) and *Saccharomyces carlsbergensis* (Wright *et al.*, 1951). This as well as some other reports that several fungi do not use pimelic acid as growth factor warrant further elucidation of the above scheme. However, species showing ability as well as inability to use desthiobiotin have been recorded, which may probably be due to blockage of the above sequence at pre and post-desthiobiotin synthesis stages respectively. Reports indicating antibiotin effect of desthiobiotin for some fungi (Lilly and Leonian, 1944)), however, remain unexplained. The suggestion that the acidic side-chain of biotin and its derivatives is an important factor with regard to their biological activity (Lilly and Barnett, 1951), and the possibility of the antagonistic effect of desthiobiotin due its longer side-chain merit attention. This is particularly relevant in view of the reported inactivity of norbiotin (with 3 methyl groups) and homobiotin (with 5 methyl group) for most fungi including *Claviceps purpurea* (Taber and Vining, 1957), *Isaria cretacea* (Taber and Vining, 1959) and *Candida albicans* (Firestone and Koser, 1960) and their antibiotin activity for *Zygosaccharomyces barkeri* (Belcher and Lichstein, 1949).

Some other biotin analogues like oxybiotin, biotinamide, N-biocytin-p-amino-benzoic acid and N-biotinyl- $\beta$ -alanine have also been used as substitutes for biotin which induced mixed response among



the fungi. While oxybiotin has only little activity for several fungi (Perlman, 1948; Firestone and Koser, 1960), the rest are at least favourable to *Saccharomyces carlbergensis* (Wright *et al.*, 1951). Several other biotin-derivatives were, however, recorded as biotin antagonists for fungi by Wright and his associates (Wright and Cresson, 1954; Wright *et al.*, 1954 b).

### Folic Acid

**Structure.** The nutritional factor was first obtained from the leaves of spinach and was accordingly designated as "folic acid" (*L. folium*). Its structure, was, however, elucidated from a sample obtained from liver, and is shown below:

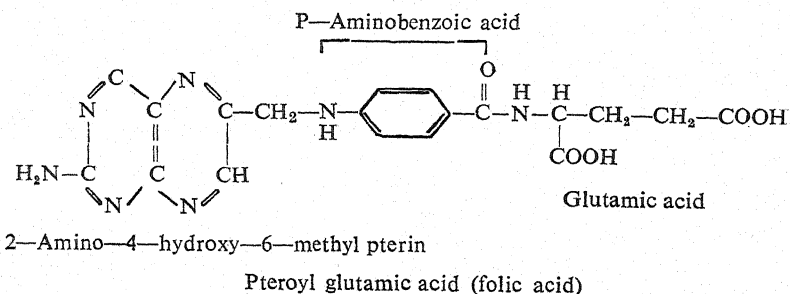


Fig. 12.8. Structure of folic acid.

The molecule consists of three different compounds, viz. glutamic acid, p-aminobenzoic acid, and a substituted pterin. Pterin and p-aminobenzoic acid are combiningly called pteric acid. Folic acid is now known to occur in different biological materials in a variety of forms, with variations in its components as well as in mode of their linkage. Some of the folic acid species contain more than one glutamic acid molecule, e.g. three in pteroyltriglutamic acid and seven in pteroylheptaglutamic acid, which are linked by  $\gamma$ -glutamyl linkages. Various other folic acids like bioppterin, rhizoppterin leucovorin are also known.

**Metabolic role.** Metabolic reactions involving various amino acids yield the so-called 1-carbon fragments which constitute a pool of reactive C-1 intermediates. These 1-carbon units are found in the form of formyl derivatives of folic acid, which act as coenzyme in reactions involving transfer of one-carbon compound during synthesis of various



cell-constituents. For example  $N^5$ ,  $N^{10}$ -methylene tetrahydrofolic acid is known to act as the coenzyme in glycine and serine interconversions. Under the catalytic influence of various derivatives of tetrahydrofolic acid, 1-carbon units play significant metabolic role (as does the 2-carbon acetyl fraction with the help of coenzyme A), and contribute to the biosynthesis of creatine, methyl nicotinamide, histidine and purine.

Role of folic acid in these biosynthetic reactions in fungi was demonstrated by Cutts and Rainbow (1950) and Nyman and Fries (1962) who reported that a mixture of amino acids and purines could substitute the requirement of p-aminobenzoic acid in some fungi.

*Fungal requirements.* Heterotrophy for folic acid as such does not seem to prevail among the fungi, and unlike bacteria, fungi generally seem to be auxoautotrophic for this growth-factor. However, the ability to synthesize folic acid is conditioned among a few fungi by availability of the folic acid precursor, p-aminobenzoic acid (PABA). This is because a few fungi, including *Rhodotorula* (Robbins and Ma, 1944; Hasegawa and Banno, 1959; Nyman and Fries, 1962; Ahearn *et al.*, 1962), a strain of *Saccharomyces cerevisiae* (Rainbow, 1948) and *Blastocladia pringsheimii* (Crasemann, 1957) have been found to be deficient for PABA and thus they require an extraneous supply of PABA for the synthesis of folic acid. PABA-deficient mutants have also been artificially induced in many fungi (Fries, 1945; Bonner, 1946; Giles, 1946; Iquchi, 1952; Pontecorvo *et al.*, 1953).

Fungi require PABA for the synthesis of folic acid but whether the role of PABA is limited to that extent only or it has some independent role in fungal metabolism is still in doubt. There are some suggestions (Cochrane, 1958) regarding PABA's independent activity also, but these are based mainly on certain reports that some fungi, like *Neurospora crassa* (Zalokar, 1948; Agarwala and Peterson, 1950) and *Saccharomyces cerevisiae* (Woods, 1954) do not utilize folic acid although PABA stimulates their growth. However, non-utilization of folic acid by some fungi may be due to their inability to cause the initial break-down of the molecule prior to absorption. Such a possibility is supported by some recent reports that folic acid is first broken down and then only the PABA part is utilized (Nyman and Fries, 1962). Moreover, some other fungi like *Rhodotorula* and *Fusarium* (Mathur *et al.* 1964) are able to utilize folic acid. Moser's (1960) findings in this regard are also very significant, that several species

belonging to the section *Phlegmacium* of *Cortinarius* are stimulated by folic acid but not by PABA. These might be very interesting situations, because they probably indicate that PABA has no direct role in cellular metabolism of these fungi. The stimulatory influence of folic acid, but not of PABA, possibly suggests that the step(s) leading to the conversion of PABA into folic acid is (are) blocked in these fungi.

### Inositol

**Structure.** Requirement of inositol as a growth factor for fungi was first demonstrated in yeast by Eastcott (1928). Incidentally inositol was the first component of the "Bioscomplex" to be identified. Inositol is a carbohydrate, with the same empirical formula as the monosaccharides *i.e.*  $(CH_2O)_n$ . It is closely allied to sugar-alcohols like sorbitol, dulcitol etc. and differs from them mainly in its cyclic form, and hence is called a carbocyclic alcohol. Of as many as nine stereoisomeric forms of inositol, only *myo*-inositol shows vitamin like activity. *myo*-Inositol, which is chemically designated as hexahydroxyl-cyclohexane, has the following structural configuration:

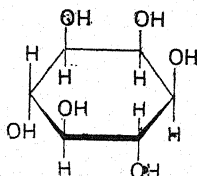


Fig. 12.9. Structure of inositol.

**Metabolic role.** The metabolic role of inositol is still obscure, although it has been suggested that it helps in the maintenance of certain cytoplasmic structures, like mitochondria (Ridgeway and Douglas, 1958). In contrast to other vitamins, inositol has not yet been assigned any coenzymatic function. Perhaps it does not have a catalytic role in cellular reactions which is also indicated by the large amount in which it is required (ca. 5 mg/l). It has often been suggested (Fuller and Tatum, 1956; Cochrane, 1958; Fries, 1965) and perhaps rightly so, that inositol may better be placed among the metabolites rather than among the vitamins. Its occurrence in plant as well as animal cells largely in association with phospholipids also suggests that it comprises structural component of the cells, rather than a functional unit.

**Fungal requirements.** Since Eastcott's (1928) observation of inositol requirements by *Saccharomyces* many strains of yeasts have been recorded with inositol deficiency. Fries (1961 a) has reviewed the literature on inositol requirements of a number of genera of yeasts, which may be partially or completely heterotrophic. *Saccharomyces uvarum* and *Schizosaccharomyces pombe* were reported to be totally deficient for this factor by Burkholder (1943) and Burkholder and Moyer (1943).

Among the filamentous fungi total or partial deficiency of inositol has been recorded in *Cercospora cruenta* (Jandaik and Kapoor, 1972), *Fusarium oxysporum* f. *cumini* (Mathur *et al.*, 1964), *Diplocarpon* (Shirakawa, 1955), *Trichophyton* spp. (Georg, 1951; Robbins *et al.* 1942), *Diaporthe phaseolorum* var. *bataticola* (Timnick *et al.*, 1951), *Colletotrichum lindemuthianum* (Mathur *et al.*, 1950), *Sclerotinia camelliae* (Barnett and Lilly, 1948), *Rhizopus suinus* (Schopfer, 1942), *Lophodermium*, *Melanconium*, *Valsa* (Fries, 1938) and *Nematospora gossypii* (Kogl and Fries, 1937). Most of the inositol requiring fungi have been found to be heterotrophic for thiamine or biotin also. Inositol-deficient mutants have also been obtained in number of fungi including *Neurospora crassa* (Bealle, 1944).

### Vitamin B<sub>12</sub> Group

**Structure.** Cyanocobalamin was the first compound of this group, to be isolated simultaneously by Rickes and his associates as well as by Smith (1948) cf. White *et al.* (1959). The complex structure of this vitamin was elucidated by Bonnet *et al.* (1955). This vitamin consists of four pyrrole rings and is thus a porphyrin. However, in contrast to other porphyrins, two pyrrole rings of this vitamin are linked directly, and only the other two exhibit the usual methane bridges in their linkages. The tetrapyrrole ring of this vitamin contains in its centre a single atom of cobalt. In cyanocobalamin, the unsatisfied valence of cobalt is filled up by a cyanide group and hence the name cyanocobalamin. Structure of some other cobalamins, like hydroxycobalamin (Vitamin B<sub>12a</sub>, B<sub>12b</sub>), nitritocobalamin (Vitamin B<sub>12c</sub>) etc. is essentially the same except that the cyanide is replaced by respective groups. However, cyanocobalamin is the usually active form of this vitamin.

**Metabolic role.** The role of this vitamin in fungal metabolism is not yet clear. Generally, it is held that vitamin B<sub>12</sub> is essential for the reduction of 1-carbon fragments, e.g. reduction of formyl to methyl group. However, its role must not be confused with that of

folic acid which helps only in the transfer of C-1 unit and not in their reduction. A derivative of this vitamin, viz. cobamide has been suggested to help in the transformation of propionyl CoA into succinyl CoA. Further, this vitamin acts as the co-factor along with ascorbic acid during the catabolic oxidation of the amino acid tyrosine into homogentisinic acid. All these possible roles of this vitamin are yet to be substantiated in fungi, which have only recently been shown to synthesize this growth factor (Tanner, 1960), and has given rise to logical anticipation of its participation in fungal metabolism.

**Fungal requirements.** Requirement for this vitamin is restricted to animals and microorganisms only. As indicated above, synthesis of cyanocobalamin in fungi is known only for about a decade, and natural total deficiency for this vitamin has been reported only in a singular case, viz. a marine phycomycete *Thraustochytrium globosum* (Adair and Vishniac, 1958). *Candida albicans* (Littman and Miwatani, 1963) is the only other fungus, which shows a partial heterotrophy for this vitamin. Stimulated mycelial growth due to vitamin B<sub>12</sub> has also been reported in *Colletotrichum capsici* (Misra and Mahmood, 1961). However, vitamin B<sub>12</sub>-less mutants have not yet been isolated or produced. *Ashbya gossypii* (Smiley *et al.*, 1951) and *Aspergillus niger* (Nicholas, 1952) were reported to synthesize vitamin B<sub>12</sub>-like substances but the method employed for identification of the vitamin in those studies are doubted (Ford and Hunter, 1955).

### Choline

**Structure.** Free choline is nitrogenous alcohol which is generally present in the cell as phosphatidyl choline or lecithins and thus constitutes major fraction of phospholipid-contents of the cell. However, as a growth factor choline may be described under vitamin B-complex. It has the following chemical make-up:

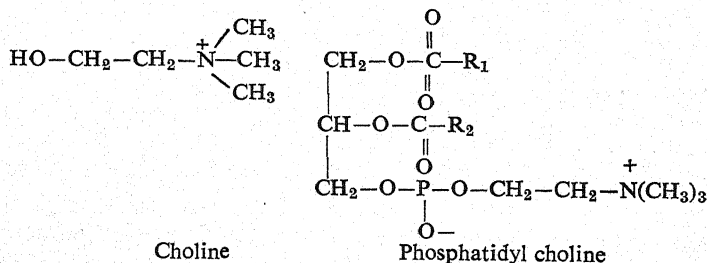


Fig. 12.10. Structure of choline.

**Metabolic role.** Phosphatidyl cholines are important constituents of biological membranes. In animals, lecithins have been found to be required for normal transport and utilization of other lipids. However, nothing is known about their activity in fungal system, and further investigation on this aspect may be rewarding.

**Fungal requirements.** Partial deficiency for choline has been observed but in a few fungi only, including *Alternaria solani* (Lewis, 1952), *Cercospora beticola* (Thind and Sharma, 1960), *C. viticola* (Sethi and Munjal, 1963), *C. cruenta* (Jandaik and Kapoor, 1972) etc. Choline is, however, required in a higher dose than most of the vitamins dealt earlier (except inositol).

### OTHER GROWTH FACTORS

In addition to the above vitamins, several other factors have been reported to exert stimulatory effects on fungal growth and activities. However, in most of the cases, the actual growth-factor or its chemical structure has not yet been elucidated and hence their mode of action also is not fully understood.

*Pilobolus* spp. are known to require some hemin derivatives (Page, 1952) like coprogen (Hesseltine *et al.*, 1952) and ferrichrome (Neilands, 1953). Coprogen has also been found to be synthesized by various other fungi (Hesseltine *et al.*, 1953) including *Penicillium* sp. (Pidacks *et al.*, 1953) and *Ustilago sphaerogena* (Gairbaldi, and Neilands, 1955). It has been suggested that ferri-chrome is a precursor of cytochrome *c* (Neilands, 1953). Another growth factor yet to be completely understood, though partially purified (Whaley and Barnett, 1963), is mycotrophein which is essential for the cultural growth of *Gonatobotrys simplex*. Mycotropheins extracted either from the host fungus or any other fungus are equally effective. Some sterols have also been reported to stimulate fungi including *Pyricularia oryzae* (Weinraub *et al.*, 1958), *Aspergillus niger*, *Torula utilis* (Jefferson and Sisco, 1961) and yeasts (Devloo, 1938; Andreassen and Stier, 1953). Even certain volatile growth factors have been suggested to occur. *Aagricus campestris* produces a volatile aliphatic hydrocarbon, 2, 3-dimethyl-1-pentene in its mycelium which is needed to stimulate its basidiospores to germinate (McTeague *et al.*, 1959). Similarly, several wood-rotting Hymenomycetes are stimulated by a volatile factor produced by wood (Suolahti, 1951). An unknown growth substance, the "M-factor", is thought to be present in the root exudates of higher plants which promotes the growth of mycorrhizal fungi. Simi-

larly, extracts of a number of other natural materials, like tomato, wood, malt, jute, yeast, coconut-milk etc. have been reported to cause stimulated response in a number of fungi, and only future investigations may reveal, whether these are due to some yet unknown growth factors or they are due to some factors already discussed.

It is well known that fungi are prolific producers of growth factors like indole acetic acid (IAA) and gibberellic acid etc. Brian (1960) found that most of the 25 soil fungi he studied were able to produce IAA. Roberts and Roberts (1939) studied about 150 different fungi, bacteria and actinomycetes, 66% of which produced metabolites with hormone-like effect on *Avena* coleoptile. However, reports concerning their effect upon fungal growth *in vitro* are conflicting and it appears that both inhibitory as well as stimulatory effect are produced on different fungi and in different concentrations. Verma (1973) observed that foliar application of gibberellic acid produced both stimulatory as well as inhibitory effect upon the rhizosphere fungal flora of *Nicotiana rustica*, depending upon the fungal species as well as concentration of the hormone. Leonian and Lilly (1937), Leonian (1938) and Bhargava (1946) also reported both stimulatory and inhibitory effect of heteroauxins on fungal growth at different concentrations. Thus, it may be interesting to investigate their effect upon the organism producing them.

## GROWTH

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Fungi exhibit remarkable diversity in form, ranging from microscopic unicellular organisation as in yeast to large macroscopic bodies as in some Basidiomycetes. A vast majority of fungi have, however, cylindrical filamentous microscopic thallus, known as the mycelium. Some fungi also exhibit polymorphism. Cochrane (1958) has listed more than a dozen fungi which are dimorphic and change from mycelial to non-mycelial phase and vice-versa. The mycelium may be simple or branched, septate or unseptate, may aggregate into complex vegetative and reproductive structures. Due to wide range of structural variations in fungi, it is difficult to give some precise definition of fungal growth. More so, because concept of growth in general is still vague. Doubts have been expressed, whether we will ever be able to understand fully the process of growth (Rahn, 1932). Thompson (1948) conceded that growth is a vague and complex phenomenon. Needham (1942) tried to comprehend most of the aspects of growth and according to his concept, growth may involve an increase in the number of nuclei, in the number and size of cells, or in the amount of non-living structural matter. Cochrane (1958) preferred to give only a workable definition that growth denotes an increase in either mass or number of cells. However, in dealing with fungi, Needham's concept of growth appears to be quite appropriate and should provide a basis for discussion on this aspect.

Different aspects of fungal growth have earlier been discussed by Lilly and Barnett (1951), Cochrane (1958), Mandels (1965), Robertson (1965) and Jerebzooff (1965).

### TYPES OF GROWTH IN FUNGI

Despite all the complexities of phenomenon of growth, it is generally accepted that the essence of growth lies in replication of living mate-

rial, or the protoplast. Under optimal conditions, replication of the protoplast leads to increase in its mass at a logarithmic rate. In fungi, replication of protoplast may have different manifestations in the mycelial and the non-mycelial types. For example, in yeast, where the protoplast is discrete and in the form of small cells, growth will mean increase in the number of independent cells. In the filamentous fungi, on the other hand, the protoplast will mainly enlarge in dimension as a consequence of growth. Following are some of the important patterns of growth, which have been recognised.

#### (a) Yeast Type Growth

This type of growth, which is mainly recorded in yeasts, is characterized by a process, popularly known as budding. In this phenomenon, the growing protoplast of the parent cell leads to the formation of a bud, which after increase in size separates to form a new cell. The cycle goes on repeating and the cells multiply in number.

#### (b) Plasmodial Growth

In this type of growth, the protoplast may replicate anywhere in the plasmodium. This implies that each small portion of the plasmodium is capable reproducing itself. However, this actually may not happen, because the entire protoplast of the plasmodium is not uniformly similar. Different portions show different density and inclusions and thus the

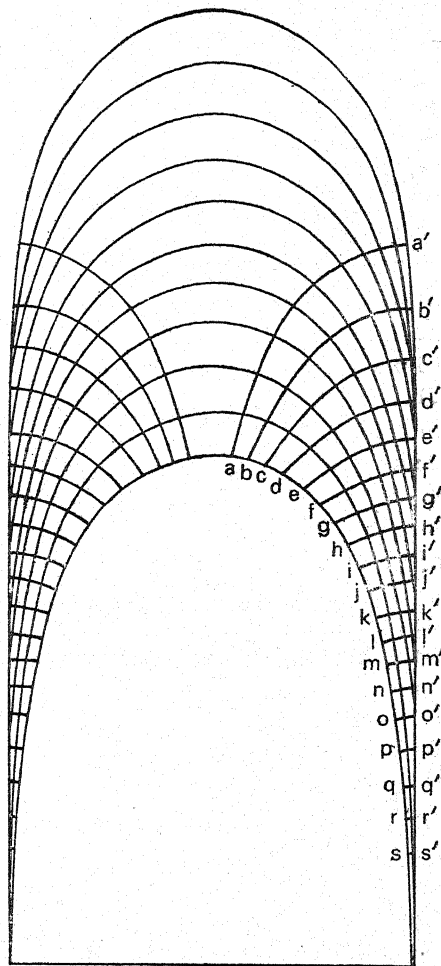


Fig. 13.1. Showing hyphal-growth in length due to intussusception of materials principally at the tip region (After Reinhardt)



replication capacity may actually remain confined to one or the other part of the plasmodium.

### (c) Apical Growth

In filamentous fungi growth is effected at the tip of the hyphae, into which new protoplast continuously streams. This type of tip-growth enables the hyphae to make continuous advance attaining newer territory. A fast growing hypha of *Neurospora* forges ahead by about 5 mm per hour. This implies that the protoplast in the older portion of hyphae must also increase at a compensatory rate to ensure its incessant streaming into the growing zone. Majority of fungi being filamentous, show this type of growth and, therefore, discussion on this pattern of growth is elaborated.

As early as 1892, Reinhardt recognized that filamentous fungi expand their thalli through hyphal tip growth and since then the view has been supported and elaborated by a large number of workers (Smith, 1923; Castle, 1958; Zalokar, 1959; Grove *et al.*, 1967, 1970; Girbardt, 1969). Reinhardt (1892) with the help of simple transformation diagrams (Fig. 13.1) concluded that the hyphal growth is accomplished by intussusception of new materials at the tip only, without any addition at the sides. Smith (1923) measured the distance between the hyphal tip and the first septum, and also the inter-septal distances to conclude in a number of fungi belonging to all the classes, that their growth was apical in nature. Butler (1958) with the help of markers also reached a similar conclusion. However, the exact site of intussusception and particularly its mechanism are yet to be fully understood.

### Site of Cellular Extension

The growth of filamentous fungi is restricted to the hyphal tip, comprising about 50 to 100 microns of apical portion, while the older part of the hypha is incapable of growth. Structural and cytochemical evidences suggest that the hyphal tip portion is distinct from the rest of the thallus (Brunswik, 1924; Girbardt, 1955, 1957; Robertson, 1958, 1959; Zalokar, 1959; 1965; Strunk, 1963, 1968; Grove *et al.*, 1967; Bartnicki-Garcia *et al.*, 1968; Brenner and Corrol, 1968; Heintz, 1968; Marchant and Smith, 1968; McClure *et al.*, 1968). Functionally, the apical portion has been further distinguished into three zones: (i) the extreme tip or apical zone, which is supposed to be primarily concerned with the cell extension and wall synthesis due to intussusception of new materials (ii) a sub-apical

zone, rich in cytoplasm and inclusions, supposed to perform other activities concerned with growth, besides translocating the requisite materials to the tip, and (iii) the distal portion, which runs basipetally from the sub-apical to the rest of the hypha and is very much vacuolated because it is thought to supply the functional cytoplasm to the portions ahead of it.

The sensitivity of the extreme tip or the apical zone of the hypha, as well as its direct involvement in hyphal growth has been amply demonstrated by manipulation studies. Castle (1942), Middlebrook and Preston (1952 a), Thimann and Gruen (1960) as well as Robertson (1958) studied the effect of various minute particles, like spores of *Lycopodium*, droplets of water or osmotic solutions etc. when they were placed on the extreme hyphal tip of various fungi. Such manipulations always caused a cessation of growth accompanied with swelling of the apex and the hypha could resume its growth only through a narrow terminal filament or through one or more lateral branches, also of smaller diameters. Cessation of growth due to high intensity light-exposure of hyphal tips (Girbardt, 1957) also suggested that hyphal apices were concerned with the apical growth.

Attempts to locate the area of intussusception at the tip, with the use of suitable stains have been made (Middlebrook and Preston, 1952 a). However, in some recent studies, both in filamentous fungi and yeasts (May, 1962 ; Goos and Summers, 1964) stains have been replaced with fluorescent antibodies, which stain cells already exposed to specific antisera. This technique has yielded interesting information in case of the yeast *Schizosaccharomyces pombe* and conidia of *Fusarium*. When conidia exposed to antiserum were germinated and then treated with fluorescent antibodies, only the spore-wall fluoresced under ultra-violet light and not the germ tube (Fig. 13.2). This indicated that the area showing fluorescence did not take part in wall-synthesis. Zalokar (1959 b) on the basis of his cytochemical studies in *Neurospora crassa* concluded that the apical growing region was about  $100\mu$  long of which the  $10\mu$  tip portion was usually devoid of cell organelles including nuclei, but filamentous mitochondria were pre-

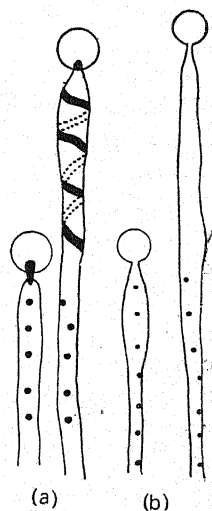


Fig. 13.2. Growth of sporangiophores of *Phycomyces*.

sent. Nuclei were, however, in large numbers in the remaining portion of apical zone. The whole apical region was found to be rich in RNA and proteins containing arginine, tyrosine, histidine and SH-groups, but was poor in glycogen. Besides, the apical portion exhibited an accumulation of the enzyme alkaline phosphatase. According to Zalokar (1965) this may be suggestive of the significance of active phosphorylation in providing the energy required for building polysaccharide bonds during wall-synthesis. The accumulation of glycogen in the sub-apical zone and its gradually diminishing quantity in the apical portion is indicative of the fact that sugar required for synthesis of wall-material is transported in the form of glycogen, which is hydrolyzed and utilized in the apical region (Zalokar, 1965).

Marchant and Smith (1968) have shown that the hyphal tips of *Fusarium culmorum* exhibit unique immunochemical properties. Recently, ultrastructural accounts of growing hyphae of various fungi have been made which reveal specialized regions in the wall (Strunk, 1963, 1968; Bartnicki-Garcia, *et al.*, 1968) as well as the cytoplasm of hyphal tips. The presence of cytoplasmic vesicles in hyphal tips and their possible role, have been recorded in quite a number of fungi. Grove *et al.* (1967, 1970) have observed the growth zone in hyphal tips of *Pythium ultimum* both by light and electron microscopy and found the apical zone 3-5  $\mu$  long. The characteristic feature of this zone was an accumulation of cytoplasmic vesicles of varying dimensions and lack of other cell organelles. However, mitochondria were marked by periodical appearance and disappearance in this region. Ribosomes, endoplasmic reticulum (ER) and dictyosomes were respectively infrequent, scarce and poorly organised in the apical zone. The hyphal wall at the apex was less than 10-20 m $\mu$  thick and was thinner than the lateral wall. Moreover, the apical wall and the underlying plasma membrane exhibited uneven profile, as compared to the lateral walls and the plasma membrane there. Similar distribution of cell organelles in the hyphae of *Phytophthora parasitica* germinating directly from the sporangium was observed by Hemmes and Hohl (1969). Presence of cytoplasmic vesicles in the apical region and their participation in the hyphal extension has also been suggested in *Ascodesmis sphaerospora* (Brenner and Carrol, 1968), *Coprinus lagopus* (Heintz, 1968), *Aspergillus niger* (McClure *et al.*, 1968), *Pythium aphanidermatum*, *Gilbertella persicaria*, *Fusarium oxysporum*, *Venturia inaequalis*, *Verticillium albo-atrum*, *Aspergillus*

*niger*, *Ascodesmis nigricans*, *Neurospora crassa*, and *Armillaria mellea* (Grove *et al.* 1970). Girbardt (1969) observed the hyphal tip growth of a number of fungi.

The sub-apical zone is characterised by abundance of protoplasmic inclusions, including nuclei, mitochondria and glycogen, but lacks vacuoles and is poor in proteins and RNA in *Neurospora crassa* (Zalokar, 1959 b). He has indicated the area of this zone as lying between 100-150 m $\mu$  from the apex. Grove *et al.* (1970) have reported the ultra-structure of this zone in *Pythium ultimum*, where the sub-apical zone extends several hundred microns between apical and distal zones. Various protoplasmic components like nuclei, mitochondria, ribosomes, ER, dictyosomes, vesicles etc. are densely packed in this zone. The cytoplasmic vesicles are in lesser number in this zone as compared to the apical zone, and are mostly clustered round the dictyosomes. There is a complete lack of vacuoles in this area. The transition from apical to sub-apical and finally to distal zone is gradual. Mitochondria are found clustered in the transitional zone between the former two.

The older portion of the hypha is marked with increasing number and size of the vacuoles with increasing distance from the apex. Ultimately, the protoplasm becomes very much vacuolated and poor in cell organelles.

### **Mechanism of Hyphal Tip Growth**

The mechanism by which the fungal hyphae shape themselves at the tip, where accretion of the structural materials takes place has been defying elucidation for a long time. Robertson (1965), sensing the complexity of the problem was perhaps right to suggest that electron microscopy could be more helping and rewarding in this field. During the last few years fungal cytologists have generally realised this need and ultra-structure of growing hyphae of a number of fungi have been studied with the help of electron microscopy. Most of the cell organelles found in other living cells have been located in fungal hyphae (Bracker, 1967), and their roles in the apical growth of hypha have also been suggested. The general pattern which has emerged till now appears not very different from other tip-growing systems, *e.g.* pollen-tubes and root-hairs of higher plants, rhizoids of some algae etc. In all such tip-growing systems, there is an apical accumulation of cytoplasmic vesicles, and it has been suggested both in case of fungi (Marchant *et al.*, 1967; Brenner and

Garroll, 1968; McClure *et al.*, 1968; Hemmes and Hohl, 1969; Grove *et al.*, 1970) as well as other systems (Rosen *et al.*, 1964; Sievers, 1967; Rosen, 1968; VanDer Woude, 1969) that these vesicles may contribute to the tip wall and provide a potential source of new plasma membrane.

However, various other organelles, like the microbodies, the endoplasmic reticulum, the lomasomes, and the plasma membrane itself, (wholly or partly) have also been considered to participate in cell-wall formation in a number of fungi as well as in higher plants. Plasmalemma was suggested as the possible site of polymerization of fibrillar polysaccharides in two cellular slime molds, *Acytostelium leptosomum* (Hohl *et al.*, 1968), and *Dictyostelium discoideum* (George, 1968). Pickett-Heaps (1967) demonstrated the important role of dictyosomes and the endoplasmic reticulum in wall synthesis of meristematic and xylem cells. Marchant *et al.* (1967) and Marchant (1968) stressed over the possible role of endoplasmic reticulum and Bracker (1967) discussed the participation of Golgi apparatus and ER in wall formation in fungi. Wilsenach and Kessel (1965) suggested that in *Penicillium vermiculatum*, lomasomes may be associated with wall formation. However, in some recent studies (Grove *et al.*, 1967, 1970; Brenner and Carroll, 1968; McClure *et al.*, 1968; Hemmes and Hohl 1969; Girbardt, 1969), a more comprehensive approach has led to the elucidation of the possible sequence of events connected with hyphal expansion, involving ER, dictyosomes and cytoplasmic vesicles, with greatest stress over the latter. The distribution of different cell-organelles in the hyphal apex, their interassociation, known functions of the endomembrane system and mechanism of growth in other tip-growing systems, helped Grove *et al.*, (1970) to formulate a hypothesis, regarding the mechanism and the participating organelles in the extension of the hyphal tip of *Pythium ultimum*. Investigations with some other fungi have also led to similar conclusions (Brenner and Carroll, 1968; McClure *et al.*, 1968; Hemmes and Hohl, 1969; Girbardt, 1969) and therefore the pattern, which seems acceptable at least under the present circumstances for a good number of fungi, is described here.

The hypothesis postulates the direct participation of ER, dictyosomes and cytoplasmic vesicles in the hyphal extension. The tip-growth of the hypha leads to continuous enlargement of its surface-area which in turn requires the formation of new plasma membrane and cell-wall extension. It is supposed that the material required

for plasmalemma synthesis, viz. lipid and protein are synthesized in the ER. ER has been recognised as the site of membrane biosynthesis and transformation in other living systems also. (Dallner *et al.* 1966; Jones and Fawcett, 1966; Orrenius and Ericsson, 1966; Siekvitz *et al.*, 1967; Silveira, 1967).

The membrane synthesis is considered to take place in the subapical zone of the hypha, as in the apical zone both ribosomes and rough-surfaced ER are infrequent, although RNA has been detected in the apical zone also (Zalokar, 1959). The subapical zone, on the other hand, is rich in most of the cell organelles, including ER and ribosomes. The membrane constituents after their synthesis in the ER are transferred to the proximal cisternae of the dictyosome. In various electron microscopic studies the ER has been seen in association with the proximal cisterna of the dictyosomes (Grove *et al.* 1967, 1968). Also, the membrane of ER and proximal dictyosome cisterna exhibited morphological similarity (Grove *et al.*, 1968). Grove *et al.* (1970) also observed that small ribosome free evagination of ER projected into the space between ER and the proximal cisterna of the dictyosome and considered that this might represent the actual transfer of membrane from ER to dictyosome through blebbing of small vesicles by the former. Vesicles of identical membrane-type have been seen to align along the developing proximal cisterna both in fungi (Moore and McAlear, 1963; Shatla *et al.*, 1966; Grove *et al.* 1970) and other lower organisms (Drum and Pankratz, 1964; Manton, 1966; Falk, 1967) and it is presumed that these ER-originated vesicles fuse and give rise to the proximal cisterna of a dictyosome (Fig. 13.3).

Transfer of the membrane components from ER to dictyosome may be achieved to facilitate the transformation of the membrane from ER-type to plasmalemma-type. Such a transformation is essential, because ER and plasma membrane appear morphologically different at high magnification (Sjostrand, 1963; Girbardt, 1965; Grove *et al.* 1968), and without this change the ER-originated membrane-components may not fuse with the plasma membrane. The dictyosomes are considered as the logical site for such a transformation. These organelles are believed to be in a state of turnover, in which cisternae are produced at the proximal pole and mature during their displacement towards the distal pole of a dictyosome (Mollenhauer and Morre, 1966 b; Silveira, 1967; Beams and Kessel, 1968). It is believed that the cisternae at the distal pole finally give rise to secre-



tory vesicles (Brown, 1969), whose contents are contributed by the luminal contents of the cisternae. The concept that the secretory vesicles are derived from and discharged by the dictyosomes is supported by various ultrastructural evidences, viz, similarity between vesicles attached to the dictyosome cisternae and those free in the cytoplasm, similarity in the staining reactions of lumina as well as membrane of distal cisternae and the vesicles attached to them (Grove *et al.*, 1970), etc. The developmental relationship between cisternae and vesicles is known in various other systems as well (Mollenhauer and Morre, 1966 a, b; Beams and Kassel, 1968).

Subsequently the secretory vesicles move from the subapical to the apical zone of the hypha. During their migration, the vesicles may increase in size, by enlargement and/or fusion with other vesicles leading to increase in their matrix material. Moreover their contents may also undergo further modification. Electron micrographs showing two or more coalesced vesicles but with their luminary inclusions still identifiable were obtained by Grove *et al.* (1970). Also, small and large vesicles exhibited similarity in their membrane types (Grove *et al.*, 1968).

Accumulation of cytoplasmic vesicles in growing tips has been reported by a host of investigators both in fungi (Brenner and Carroll, 1968; McClure *et al.*, 1968; Hemmes and Hohl, 1969; Heintz, 1968; Grove *et al.*, 1967, 1970; Girbardt, 1969) as well as in other living systems (Rosen *et al.*, 1964; Bonnet and Newcomb, 1966; Sievers, 1967; Rosen, 1968; VanDer Woude, 1969). Their predominance in growing tips suggest their role as sources of new cell surface. Further, their similarity with the plasma-membrane (Grove *et al.*, 1968) shows that they are compatible to fuse with the latter and contribute in its expansion. However, actual fusion of the two has not been observed which is attributed to the instantaneous nature of this phenomenon. Thus, a minimization in vesicle accumulation in the hyphal tip may suggest their rapid incorporation in the plasma membrane, while their greater concentration suggests the *vice versa*. Hemmes and Hohl (1969) in *Phytophthora parasitica* observed numerous vesicular inclusions in the basal plug of the sporangium and in the germination wall but none in the hyphal tip. The possible explanation proposed by them considers that the wall at the hyphal tip forms as the cell elongates rapidly, and the rapidly expanding plasmalemma might readily incorporate the vesicles arriving at the tip. On the contrary, plasma membrane does not expand as the wall is deposited in the

basal plug and germination wall of the sporangium, leading to an accumulation of the vesicles arriving at the site. Grove *et al.* (1970) have also tried to estimate the rate of vesicular incorporation in a growing hyphal tip of *Pythium ultimum*. According to their calculations, approximately 1,000 large vesicles ( $150 \mu$  diam.) and 9,000 small vesicles ( $50 \mu$  diam.) will be sufficient for one minute of hyphal ( $5 \mu$  diam.) growth, during which about  $262 \mu^2$  surface of new plasma-membrane will be assimilated.

The vesicles fusing with the plasma membrane discharge their matrix material outside the protoplast into the wall region. Grove *et al.* (1970) observed that the apical wall of the hypha of *Pythium*

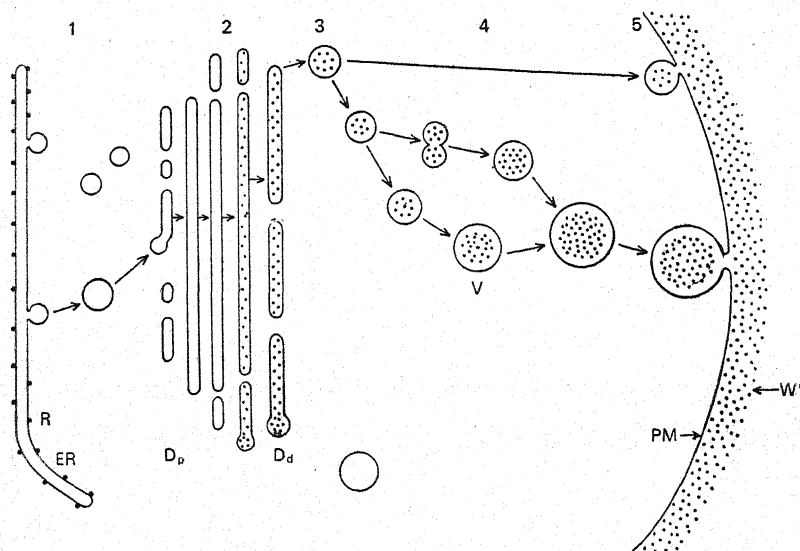


Fig. 13.3. Diagrammatic representation of the sequence leading to hyphal extension at the apex. After Grove *et al.* (1970). ER—Endoplasmic reticulum, R—ribosomes, D<sub>p</sub>—proximal pole of dictyosome, D<sub>d</sub>—distal pole of dictyosome, V—cytoplasmic vesicles, W—wall, PM—plasma membrane.

1. Transfer of membranal components from ER to dictyosome by blebbing and formation of proximal cisterna of dictyosome.
2. Transformation of cisternal contents and membrane during displacement of cisternal to distal pole of the dictyosome.
3. Formation of cytoplasmic vesicles from distal cisterna.
4. Migration of vesicles to the apex.
5. Fusion of the vesicles with the plasma-membrane and discharge of their contents to wall-region.



*altimum* possessed lumps, which resembled the contents of the vesicles. Thus it may possibly be concluded that the vesicles also transport some wall-materials and contribute to wall-extension also. Similar conclusions were arrived at by Hemmes and Hohl (1969), who reported that in direct germination of *Phytophthora parasitica* sporangia, the dictyosome-derived vesicles seem to function as a transport system for wall precursor material, probably a glucan. During indirect germination, on the other hand, the vesicles mainly contribute to the plasmalemma of the developing zoospores. Grove *et al.* (1970) have assigned yet another function to these vesicles, namely that they may also transport some wall-softening material and help in branch initiation. However, their assumption is based merely on accumulation of vesicles at potential sites of branch initiation, and therefore further observations are needed to substantiate this possibility.

The overall-mechanics of hyphal tip extension is represented in Fig. 13.3.

### Kinetics of Growth

A growing organism is a changing entity, and attempt to estimate its size and composition at a particular time requires a knowledge as to how it is changing, in other words, its growth-rate. Growth-rate of fungi may be measured in terms of changes in number of cells, in linear dimension, in cell-mass, in cell-volume, in total activity of any metabolic process, or in amount of some cellular component. Of all these methods, the most widely used is the one in which dry-weight of the cell-mass is measured. Methods employing cell-number and cell-volume have limited application, mostly confined to unicellular fungi, like yeasts. Determination of metabolic activities or amount of some cellular component provide only indirect methods for growth measurements. Mandels (1955) has discussed in detail the various methodology of measuring fungal growth and needs no further elaboration.

Whatever be the method employed, the quantitative study of fungal growth has not been as simple as that of bacteria, particularly in case of filamentous fungi. The unicellular fungi, albeit, follow generally the same pattern of growth as do the bacteria, and a typical exponential growth curve is obtained in their case. They exhibit also the same growth phases, including (i) Lag-phase, during which cells do not divide, although cell-enlargement and enhancement in their metabolic activity may occur; (ii) acceleration phase, during which cell-division

starts and increases to a level, characteristic of the next phase; (iii) logarithmic or exponential phase, marked by cell-division as a uniform and maximum rate; (iv) deceleration phase, during which rate of cell division shows a decline; (v) stationary phase, an apparent cessation of cell-division, but in fact this may represent only a balance between cell-formation and death of old cells; (vi) phase of decline or death, during which an actual decline in the number of cells takes place.

The various phases of growth may be recognised in case of filamentous fungi also, but since their growth is not autocatalytic and is restricted to the hyphal tips only, they do not actually manifest any exponential phase of growth. This phase may at best be described as phase of rapid growth. However, Plomley (1959) demonstrated that an entire hypha of *Chaetomium globosum* alongwith its branches grows exponentially; he also recorded exponential increases in the radius of a young colony. Later, growth-rate of central hyphae of the colony declined, but the marginal hyphae grew as earlier. Thus the phases recognizable in filamentous fungi may be designated as (1) lag-phase, (2) linear phase, with rapid and almost linear-growth-rate, and (3) decline phase, marked with decrease in dry-weight of the mycelium owing to autolysis.

### Growth Rhythms

Most of the organisms exhibit rhythmic changes in their growth and activities, which may either be ascribed to periodical fluctuations in external physical conditions (exogenous rhythms), or may be due to inherent characteristics of the organism (endogenous rhythms). In fungi, the most common rhythmic expression is manifested on solid media as regularly spaced concentric circles of growth, called zonations. Such zonation-rhythms may affect a fungus in a variety of ways, like its mycelial thickness, sporulation pigmentation, branching, etc., and may be of exogenous or endogenous nature. Under uniform external physical conditions, no fungus has been reported to exhibit exogenous zonation-rhythms, while the fungi which are known to produce endogenous zonation rhythms, exhibit this characteristic under certain conditions. Jerebzooff (1965) has described different aspects of the rhythmic changes in fungi.

### Factors Influencing Growth

In addition to the nutritional requirements which have been dis-

cussed in detail in specific chapters, the fungi require certain optimum physical environment for their growth and metabolism. Some of the physical factors affecting fungal growth include temperature, moisture, pH, light radiation, etc. It has generally been observed that for most of these factors fungi exhibit minimum, a maximum and an optimum requirement. Obviously the optimum requirement supporting best fungal growth is usually a small range, instead of a particular point. The minimum and maximum requirements, on the other hand, represent specific points, beyond which no growth is possible for a particular fungus and the range delimited by these two points indicates the overall requirement of a factor for that fungus. The minimum, the maximum and the optimum points are the cardinal points, which serve to indicate the growth requirement of a fungus for a particular factor. However, it must be borne in mind that growth is an overall cumulative expression of a variety of metabolic activities, including a large number of enzymatic reactions, each of which may have its own requirement of physical conditions. In fact, different cell-processes or even reactions have widely different physical factor requirements. Therefore, the optimum requirement for growth in general is obviously a point at which the largest number of reactions or processes occur either at optimum or atleast at a favourable rate. Besides, requirement of one particular factor may be governed by other factors as well.

*Temperature.* Our universe shows a temperature range of thousand of degrees, but life in any form can exist only within a tiny range of about 300 degrees centigrades, between  $-200^{\circ}$  and  $100^{\circ}\text{C}$ . In fact, for most of the species and their activities, the range is far more narrower. Fungi, in general cease to grow at temperatures above  $40^{\circ}\text{C}$  and sooner or later die depending upon the time of exposure. Below  $0^{\circ}\text{C}$  their activities stop, but they somehow manage to survive. The range of temperature for fungi is evidently narrower than that for bacteria. The optimum temperature for fungi never lies midway between the minimum and the maximum points. In other words, the fungal activity neither increases in conformity with the rise in temperature from minimum to optimum point nor it decreases uniformly as the temperature rises from optimum to maximum point. Yet, a normal temperature-growth curve shows steady increase in growth as the temperature advances from the minimum to the optimum, where the growth-attained is maximum. During the small optimal range, the growth remains at a steady state, but any further increase in temperature initiates a decline, which continues until the maximum tempe-

perature is reached, beyond which the fungus may actually die. Cardinal temperatures of a large number of wood-decaying fungi were recorded by Cartwright and Findlay (1934), which appears to be the most extensive investigation on this aspect to-date. Their data as well as similar data from various earlier authors have been presented by Wolf and Wolf (1969). Reviews on effect of temperature on fungal growth have been presented by Hawker (1950) Cochrane (1958) and Deverall (1965).

Although general pattern of temperature requirement of fungi appears to be identical yet there are considerable variations with regard to cardinal points even among the species of the same genus. Most fungi have their minimum temperature requirement between 0 to 5°C, but a few food-spoiling fungi, like *Cladosporium* and *Sporotrichum* have the capacity to grow well below the freezing point, and their minimum temperature lies between -5 to -8°C. Similar exceptional requirements have been recorded for maximum temperature also. While most fungi are unable to grow above the maximum temperature of 35° to 40°C, yet there are certain thermophilic species, which may grow well beyond these limits. Weimer and Harter (1923) observed that *Rhizopus chinensis* had an optimum temperature for growth at 40°C and a maximum temperature of 45-50°C. Similarly, Fries (1953) recorded 40°C as the optimum and 44°C as the maximum temperatures for *Coprinus fimetarius*. Otherwise, the general optimum range for many fungi lies between 15-30°C.

Fungal growth usually ceases above and below their maximum and minimum temperatures. However, their thermal death points are generally reached slightly beyond these limits. Ames (1915) found that the fungi studied by him had generally their thermal death points between 47° to 60°C, when exposed to moist heat. However, there are certain exceptional cases, where much higher temperatures are required to kill the cells. Hull (1939) found that 20% of the ascospores of *Byssochlamys fulva* survived even after 10 minutes exposure to 85°C. Similarly various wood-rotting fungi were capable of withstanding temperature as high as 105°C, and they were killed only after 12 hours of exposure at this temperature (Snell, 1923; Findlay, 1934).

Survival and death on exposure to extremely low temperatures have also been studied, but the reports available show some variations. Forbes (1939) found that spores of *Puccinia coronata* were killed after exposure to -10°C, to those of *P. dispersa* survived at -5°C for 10 minutes, and died when exposed for 4 hours (Ward, 1902). It has also been reported that spores previously stored at room temperature

or at above zero temperatures for a few days could survive subsequent exposure to subzero temperatures like  $-20^{\circ}\text{C}$ ,  $-29^{\circ}\text{C}$ , etc. (Melander, 1935; Harter and Zaumeyer, 1941). Similarly fungal cells may be kept viable at sub-zero temperatures, if they are rapidly cooled and warmed (Cochrane, 1958), which avoids the cell damage done during slow freezing and thawing.

Cardinal points of some fungi are tabulated in Table 13.1.

TABLE 13.1

SHOWING MAXIMUM, MINIMUM AND OPTIMUM TEMPERATURES  
FOR GROWTH OF SOME FUNGI

Fungi	Temperature			Authors
	Max.	Min.	Optimum	
<i>Alternaria citri</i> and <i>A. tenuis</i>	$35^{\circ}\text{C}$	$15^{\circ}\text{C}$	$25^{\circ}\text{C}$	Hasija (1970 a)
<i>Curvularia pallescens</i>	$35^{\circ}\text{C}$	$15^{\circ}\text{C}$	$30^{\circ}\text{C}$	Hasija (1970 b)
<i>Myrothecium roridum</i>	$35^{\circ}\text{C}$	$10^{\circ}\text{C}$	$25^{\circ}\text{C}$	Chauhan and Suryanarayana (1970)
<i>Aspergillus flavus</i>	$37^{\circ}\text{C}$	$15^{\circ}\text{C}$	$30^{\circ}\text{C}$	Tandon and Chauhan (1955)
<i>A. tamarii</i>	$37^{\circ}\text{C}$	$15^{\circ}\text{C}$	$30^{\circ}\text{C}$	
<i>Fusarium coeruleum</i> str. 1 & 2 str. 2	$32^{\circ}\text{C}$	$12^{\circ}\text{C}$	$24^{\circ}\text{C}$	
				Agarwal (1957)

**Hydrogen Ion Concentration.** Fungi generally utilize a substrate in the form of solution, and only if the reaction of the solution is conducive to fungal growth and metabolism. Any acid, base or salt in solution owes its chemical activities to its ions. Experience with fungi have generally indicated that they are more tolerant of acid ions ( $\text{H}^+$ ) than of basic ions ( $\text{OH}^-$ ). However, most fungi grow at pH between 4 and 8, although there are some exceptions which have either a narrower or a wider range of tolerance (Tandon, 1961).

Most of the investigations on pH-requirements of fungi have provided only limited informations concerning the pH-range for growth and sporulation of some specific fungi. Practically, no attempt to diversify such studies to pH-requirement for various metabolic processes of fungi, has been made as yet. Little attention has also been paid to the changes in the internal pH of the fungal mycelium as well as pH-relations of phytopathogenic fungi and their hosts.

pH of the surrounding medium exerts profound influence upon the availability of certain metallic ions, which may at specific pH, form

insoluble complexes. Metals like, magnesium, iron, calcium and zinc are available to the fungus at low pH, but they become insoluble at higher pH. Narsimhan (1969) found that utilization of nitrate nitrogen as well as inorganic ammonical nitrogen by *Sclerotium* spp. was pH-dependent.

pH of the substrate also affects the permeability of the cell-membrane, internal pH of the mycelium and the enzyme-activities. At acidic pH, the cell membrane becomes saturated with ( $H^+$ ) ions, which limit the passage of essential cations. The reverse condition obtains when the medium is alkaline and the accumulated ( $CH^-$ ) ions prevent the passage of essential anions.

Enzyme activity is also known to be conditioned by the reaction of the medium. Although different enzymes have different pH-optima for their activity, the general favourable range lies between pH 4 and 8.

Another aspect of hydrogen ion-concentration and fungal growth is regarding their pH-altering effects upon the media in which they grow. Such an effect is caused due to uptake or release of anions or cations from or to the media. If this aspect of fungal nutrition is left uncared for, it ultimately leads to the cessation of growth and metabolism and causes the death of the fungus. Excretion of different metabolites of sugars, like organic acids, amino acids, carbon dioxide (which dissolves in the medium to form carbonic acid) etc. all contribute to the pH changes of the medium. Such an effect of fungal metabolism is of course resisted and delayed by the use of appropriate buffers in recommended doses, but is hard so be entirely prevented. Of course, the technique of continuous culture and use of flowing media (L. Fries, 1956 a) may help to get rid of this problem.

pH-optima and pH-range of a few fungi are tabulated in Table 13.2.

Several investigators have reported double pH-optima for growth of a number of fungi (Robbins, 1924; Scott, 1924; Mathur *et al.* 1950; Fries, 1956; Grover *et al.* 1970). This phenomenon has been explained on the basis of reduced activity of some enzymes, or interference with the absorption of some inorganic ions at that pH due to chelation, leading to reduced growth between the two pH-optima (Lilly and Barnett, 1951; Cochrane, 1958).

**Visible Light.** Various fungal processes, including mycelial growth, sporulation, spore-germination etc. are influenced by visible radiations (400-800 m $\mu$ ). Effect of light on reproductive processes has been discussed in Chapter XIV.



TABLE 13.2

SHOWING pH-OPTIMA AND pH-RANGE FOR GROWTH OF SOME FUNGI

Fungi	pH-range	pH-optima	Authors
<i>Diplodia typhina</i>	6.1-9.0	7.6	Tandon and Srivastava (1963)
<i>Botryodiplodia theobromae</i>	6.1-9.0	7.6	Tandon and Srivastava (1963)
<i>Phoma destructiva</i> Iso. 1	2.0-9.0	6.5	Grover <i>et al.</i> (1970)
" " Iso. 2	2.0-9.0	3.0	" " " "
" " Iso. 3	2.0-9.0	7.0	" " " "
<i>Myrothecium vridium</i>	2.90-7.95	6.0	Chauhan and Suryanarayan (1970)
<i>Alternaria citri</i> and <i>A. tenuis</i>	2.7-8.0	5.4	Hasija (1970 b)
<i>Curvularia pallescens</i>	2.7-8.0	5.4	Hasija (1970 a)

That light exerts both stimulatory and inhibitory influence on mycelial growth is well recorded, but the exact mechanism involved in this process is far from understood. Page (1965) distinguished two types of light-effect on developing fungal mycelium, *viz.* (i) non-oriented response, which includes stimulation or inhibition of rate of mycelial growth or synthesis of a compound, without any spatial relationship to the direction of the light-rays, and (ii) oriented response of the mycelia to light, which bears a distinct spatial relationship to the source of the illumination. Further the oriented response to light may either be pertaining to movement, which is referred to phototaxis, or it may be in relation to growth, when it is called phototropism.

*Non-oriented mycelial response to light.* Reports of such type of response of vegetative hyphae are most infrequent and those available present varying data. Cantino and Horenstein (1956) observed that light stimulated the growth of *Blastocladiella emersonii* to as much as 141 when grown on a complex culture medium. However, response was less marked on a synthetic medium (Cantino, 1959). Similar data have been recorded by Goldstein (1963) for *Thraustochytrium roseum*. Stimulated growth of *B. emersonii* in light has been ascribed to increased CO<sub>2</sub> fixation, and also that light somehow enhances the reductive carboxylation of succinic acid to  $\alpha$ -ketoglutaric acids (Turian and Cantino, 1959; Cantino and Turian, 1961). Only blue light

stimulates the growth, in which role of some photo-receptor has also been suggested but no such compound has yet been identified.

Another set of data indicate light as an inhibitory factor for growth of fungi. Many earlier investigators reported growth of fungal hyphae, when incubated in strong light (Borriss, 1934; Harter, 1939; Jensen, 1941; Chowdhury, 1944; Page, 1952 a). *Sclerotinia fructigena* and *Karlingia rosea* attained lower mycelial dry-weight when incubated in light (Hall, 1933; Haskins and Weston, 1950), while growth of *Botrytis squamosus* was completely stopped (Page, 1956). In many of these investigations, growth was resumed when the fungus was transferred to darkness, which suggests that exposure to light does not induce any irreversible change. However, there are some indications that light induces certain metabolic changes in fungi. Exposure to light is supposed to cause the synthesis of certain pigments. For example, it is a common observation that *Neurospora* culture grown in light becomes pink, which according to Zalokar (1954) may be due to an enhanced carotenoid synthesis. Stimulated melanin synthesis occurred when *Cladosporium mansonii* (Sussman *et al.* 1963) and *Aureobasidium pullulans* (Lingappa *et al.* 1963) were grown in light. On the contrary, Schaeffer (1953) observed that a black mutant of *Neurospora crassa* failed to synthesize melanin, when exposed to blue light. Other synthetic activities are also reported to be influenced by light. A common response to light-exposure seems to be stimulated wall synthesis.

*Oriented mycelial response to light.* Oriented mycelial response to light may be (a) phototactic or (b) phototropic. Phototropism is definitely the more common phenomenon among fungi, and has received greater attention. Fungi belonging to diverse taxonomic groups are known to exhibit phototropism, but it is mostly confined to reproductive structures and only a few reports on vegetative mycelial response of this kind are available. Gettkandt (1954) has studied in detail the phototropic responses of germ tubes in some fungi and has observed negative phototropic curvatures in four out of seven species of *Puccinia* studied by him. The phenomenon has been explained on the basis that light exerts an inhibitory effect upon elongation of the wall farthest from the light-source.

Phototactic response to light is rather unknown in fungi, although it may be interesting to investigate such a possibility atleast in the swimmers of some aquatic fungi, particularly those, which possess eye-spots. Positive phototactic response has been recorded in the migrating pseudoplasmodia of *Dictyostelium* spp. which are extre-



mely sensitive to light stimulus (Bonner and Shaw, 1957; Bonner, 1959), although the mechanism involved is not yet understood. The mechanism of action of light upon fungal growth and activity in general is far from clear, and the existence, nature and precise location of photoreceptors, if any, are not yet free from controversy.

*Ultraviolet and Ionizing Radiation.* Fungi manifest two kinds of responses to ultraviolet radiations, viz. (i) mutation and (ii) death, depending upon the doses as well as the wavelength. The most effective lethal as well as mutagenic wavelengths are in the region of 260-265 m $\mu$ . It has been observed that the shorter wavelengths are more lethal than the longer ones, which are more effective as mutagenic and cause non-lethal mutations. Conidia of *Aspergillus terreus* mutated when exposed to higher wavelengths, 297-313 m $\mu$ , or to sunlight (Hollaender and Emmons, 1939). Still higher wavelengths are tolerated by most organisms. On the contrary, wavelengths shorter than 200 m $\mu$  appear to be highly lethal, although such wavelengths have seldom been employed. The lethal effects of these wavelengths have been ascribed either to induction of recessive lethal mutations or to non-genetic damage to nuclei (Norman, 1951). However, such generalisation regarding the first mechanism seems to be over-simplification, because effect of radiation cannot be expected to be the same in case of a multinucleate conidium or mycelium and a uninucleate conidium. Moreover, radiation is known to cause several cytoplasmic responses as well.

Whether mutagenic or lethal, a generalisation which is usually acceptable with regard to UV radiations and fungi is that those wavelengths which are absorbed by the nucleic acid are usually more effective. Evidently, the absorbed radiations cause such chemical changes in the nucleic acid components of genes and chromosomes, which in extreme cases prove lethal, otherwise they are manifested as non-lethal mutations. Fungi showing such correlation generally exhibit identical action spectra with approximately 260 m $\mu$  wavelengths as the most effective. Pomper and Atwood (1959), as well as Pomper (1965) have enlisted several fungi showing the "nucleic-acid" type action spectra. This is suggestive of the fact that UV-radiation generally influences fungi through nucleic acids in general and deoxyribo-nucleic acid in particular. The pyrimidine bases (thymine and cytosine) of the DNA molecule were found to be more sensitive than the purines (adenine and guanine) (cf. Deering, 1962), and it appears that the absorbed radiation causes the formation of a thymine dimer in the DNA molecule. This gets support

from the finding of an enzyme by Wulff and Rupert (1962), which was effective in splitting the thymine dimer. This light-stimulated enzyme thus seems to be functional in getting rid of the reversible effects of UV-radiation in light.

Various non-genetic effects of ultraviolet radiations have also been recorded in fungi, including delay in budding in yeast (Wyekoff and Luyet, 1931; Oster, 1934 a, b, c), spore germination in *Rhizopus* spp. (Dimond and Duggar, 1940 a, b; Taguena, 1959), etc. Svihla *et al.* (1960) observed that UV-irradiated cell of *Candida* sp. collapsed with apparent precipitation of the cytoplasm. However, observations of Townsend and Sarachek (1953) on UV-radiated baker's yeast (*S. cerevisiae*) were quite different, including loss of shape as well as development of granular cytoplasm and giant cells. Savulescu and Tudosescu (1968) reported that single UV-exposures of 15-30 minutes stimulated mycelial growth of *Aspergillus niger* and *Botrytis cinerea*, but prolonged irradiation for 1-2 hour retarded the growth of *A. niger* and *Fusarium* sp., and caused loss of vitality in *B. cinerea* and *T. roseum*.

A few more effects of UV-radiation have been recorded, of which stimulatory effect of near-ultraviolet radiation (310-400 m $\mu$ ) upon sporulation (Leach, 1962; Leach, 1967) has proved a useful tool in the hands of mycologists in general and mycophysiologist in particular.

*Ionizing radiation.* Only few non-genetic effects of ionizing radiations on fungi have been recorded. X-ray exposure retarded cell-division and budding in *Saccharomyces cerevisiae* (Holweck and Lacassagne, 1930; Burns, 1956). Conidia of *Aspergillus niger* exposed to X-rays exhibited higher catalase activity (Tai, 1962), but that may also be attributed to production of H<sub>2</sub>O<sub>2</sub> in the medium itself due to irradiation. X-rays have also been suggested to cause permeability changes, influence phosphorus metabolism and enhance amino acid content of protoplast (cf. Pomper, 1965).

On the contrary, reports of genetic effects of ionizing radiations abound. Although, various types of ionizing radiations including X-rays,  $\gamma$ -rays,  $\alpha$ -rays,  $\beta$ -rays etc. have been employed in such studies, it appears that in general densely ionized radiations are more lethal, while more dispersed radiations are mutagenic (Stapleton *et al.* 1952). The different types of ionizing radiations differ in their energy, penetration as well as the extent of ionization they cause. Otherwise, as their name indicates, all such types of radiations have a common

mechanism of action in that they all produce ion-pairs in the material they pass through.

The effectiveness of X-ray radiations generally increases linearly with the dose and usually an exponential or near exponential dose-response curve is obtained (Arima, 1951 b; Iguchi, 1951). However, there are some reports of sigmoidal survival curves also (Luyet, 1932; Ford and Kirwan, 1949), but various reasons including presence of more than one nuclei per cell have been assigned (Pomper and Atwood, 1955). Recently Mirchink *et al.* (1968) have concluded that dark-pigmented fungi are more resistant to radiations. In a comparative study they found dark-pigmented *Pleospora herbarum* more resistant than the light pigmented *Cladosporium cladosporioides* to  $\gamma$ -radiations. They suggested that such inherent resistance to radiations might be the principal factor governing greater distribution frequency of coloured forms at higher altitudes.

Mutagenic effects also increase with the dose of ionizing radiation applied, but to a certain extent only, beyond which the mutagenic efficiency does not increase and remain almost static.

*Relative humidity.* Fungi in general require high moisture level for their growth and metabolism. Free water is although essential for most fungi, a few higher forms may do well without it. A high relative humidity is, however, equally desirable for all the groups of fungi. Generally 95 to 100% relative humidity supports best growth of most fungi and those below 80 to 85% are inhibitory for them. Some exceptional ones are able to thrive even at 65% level of humidity (Snow, 1945). Fungi like *Stereum frustulosum*, *Schizophyllum commune* and *Aspergillus* spp. show a medium requirement and thrive at 85 to 90% humidity. Owing to their hydrophilic nature fungi generally attack only those substrates, which are rich in moisture. Under suitable moisture conditions, fungi are able to survive long even without nutrition. Vanden Berg and Lentz (1968) observed that mycelia of *Botrytis cinerea* and *Sclerotium sclerotiorum* under storage could survive without nutrient for 12 months at 95-100% R.H. In normal conditions of nutrition, the fungi require over 93% R.H. In a further study, Vander Berg and Yang (1969) correlated the moisture requirements of *B. cinerea* and *S. sclerotiorum* with their enzyme producing capacity. Production of extracellular pectolytic enzymes was greater at 94-96% R.H. than at 98-100% R.H.

Most of these moisture requirement of fungi are generally met under cultural conditions, where water is available in agar media bound within a gel, and the relative humidity is nearly 100% in

culture flasks, or petri-dishes. Thus, it is in the natural environment, where the fungus has to face a testing time in want of favourable humid conditions.

**Chemicals.** Several chemical factors are known to exert their influence on fungal growth. Effect of some growth promoting substances like vitamins, auxins etc. have been discussed in a separate chapter. Chemical growth inhibitors include the multitude of fungicides, antifungal antibiotics, heavy metals, respiratory and enzymic inhibitors etc. Most of such inhibitors according to Horsfall (1956) possess two basic characteristics, viz. (i) they have the ability to react with an essential cell constituent and (ii) they are able to enter into the cell and reach the actual reaction-site. However, different fungistatic or fungicidal substances have different capabilities with regard to both the requirements noted above. Thus, the inhibitors may have (i) intracellular, (ii) cell-surface or (iii) extracellular site of action. Of course, majority of the antifungal substances act within the cell and inhibit vital cellular processes. Thus, they are not only able to enter into the cell, but they even reach the subcellular components associated with specific metabolic processes. To achieve this characteristics these inhibitors possess high fat solubility since the concerned cell components contain much of lipoidal materials. Example of intracellular fungicidal substances are captan, dichlone etc.

It is obvious that inhibiting substances with low fat solubility will face difficulty in reaching the subcellular components inside the cell. Hence, inhibitors of this class are believed to act at the cell-surface. Their toxic action may be related with their electronegativity and their fungistatic effects may be correlated with their nonspecific reactions on or outside the cytoplasmic membrane (Somers, 1961). Examples of such chemical substances are toxic metals.

The principal mode of action of the third group of inhibitors appears to be through the inactivation of extracellular enzymes. An aliphatic amine was found to inhibit the growth of the fungus *Lentinus lepideus*, when it was grown with cellulose as the carbon source (Finholt *et al.* 1952). When the carbon source was replaced with glucose, the fungus grew normally. It was, therefore, suggested that the inhibitor possibly acted against the extracellular cellulase enzyme. Similarly, many oxidized and polymerised polyphenols inhibit the extracellular pectolytic enzymes.

Chemical inhibitors also differ in their level of action. While majority of them act at the enzymic level and influence vital cellular processes, the level of action of some fungicides is not yet ascertained.

Several fungicidal substances produce morphological abnormalities in fungal mycelia. The antifungal antibiotic griseofulvin is a good example of such substances. Grove *et al.* (1952) recorded that griseofulvin in low quantities caused hyphal distortion, which may indicate that this antibiotic interferes with the wall-synthesis. However, it appears to be specific inhibitor of fungi with chitinous cell-wall only (Brian, 1960) and that way its action is analogous to that of penicillin against bacteria.

Fungicides and their morphological and physiological action have formed the substance of many plant pathological texts and monographs (Horsfall, 1956; Martin, 1959; Horsfall and Dimond, 1960) and is obviously beyond the scope of this book. Inhibitors active against enzyme synthesis and activity have been discussed earlier.

## PHYSIOLOGY OF REPRODUCTION

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Reproduction in fungi is accomplished by various types of specialized bodies which have been designated by different names depending on their morphology, and functional behaviour. The morphology of the reproductive bodies as well as their mode of development also serve as the basis of fungal taxonomy. The fungi reproduce vegetatively or by spores. Reproduction by means of spores has, therefore, been designated as sporulation. The spores are minute separable bodies with characteristic configuration and colour. Their morphology varies with the organisms. There are two major types of spores, (i) spores produced during asexual reproduction, and (ii) spores produced as a result of sexual reproduction. In nature, the asexual reproduction dominates over the sexual reproduction. Therefore, generally the term sporulation in literature refers to the production of asexual spores. The asexual spores may be produced within the sporangium. When they are non-motile, they are designated as sporangiospores. The motile spores produced in sporangium are referred as zoospores. In higher fungi, the spores are produced on specialized structures known as conidiophores and, therefore, they are generally referred as conidia. The conidiophores, may be developed within or upon specialized bodies like pycnidium, acervulus, sporodochium, synnemata, etc. The asexual spores usually lack vacuoles and the protoplasm is more concentrated. Their viability is short and they are capable of rapid germination. Such fungi, which produce only asexual spores and in which sexual phase is unknown, are placed in a rather synthetic group, the Fungi Imperfecti.

A large number of fungi are also capable to produce another type of spore which are developed after the nuclear fusion. In simpler fungi this nuclear fusion is accomplished by union of two nuclei from two different gametangia, which can generally be distinguished morphologically. The resulting zygote is, therefore, produced as



a result of sexual reproduction. In higher fungi, the fusion is not usually a clearly sexual one, but takes place between two nuclei derived from a self-fertile mycelium (homothallic) or from two self-sterile mycelia (heterothallic). These nuclei may pair, but the fusion is generally delayed. This phase is known as dikaryotic phase and is quite common among the Ascomycetes and Basidiomycetes. Eventually, nuclear fusion takes place, and is followed by meiosis and formation of spores. Such spores, though derived as a result of sexual reproduction, yet generally their designation, in literature as "sexual spores" has been avoided. They are usually referred as perfect spores to distinguish them from the imperfect spores or the asexual spores.

## FACTORS

Physical and nutritional factors have a pronounced effect on sporulation of fungi. Temperature, light, hydrogenion concentration, aeration and humidity are some of the important physical factors, which influence fungal reproduction. Nutritional factors like the sources of carbon, nitrogen, vitamins and trace elements also determine the rate of spore development under natural conditions. The effect of some such factors is detailed below:

### Temperature

In nature, temperature not only determines the production of spores, but it is also a decisive factor in their dissemination and geographical distribution. The range of temperature for reproduction in fungi is comparatively much narrower than that for growth. Production of asexual spores is usually attained in wider limits than the perfect spores. Influence of temperature on sporulation has been studied on diverse groups of fungi. Most of the work has, however, been carried out under laboratory conditions. Under natural conditions, fungi have to face considerable fluctuation of temperature. The spore-production, however, is generally optimum between 25 to 28°C. The temperature-curve of reproduction for most of the fungi is generally similar to growth-curve and optimum is usually closer to the maximum. The relatively narrower range of temperature permitting reproduction suggests that this phase involves some physical and chemical processes which are not necessary for vegetative growth and which are more exacting in their temperature-requirement than those which suffice for vegetative phase. Hawker *et al.* (1957) made

interesting observations with *Rhizopus sexualis* and some other Mucorales. Initiation of zygospores in these fungi, is inhibited at 7-10°C (exact temperature varies with the species). Slow mycelial growth and sporangial production, however, continues. Zygospores, which have passed early stages of development at a higher temperature, however, continue to develop even if they are then placed at a temperature low enough to prevent initiation. Hepden and Hawker (1961) further noted that when a current of air was passed over a mature colony bearing numerous zygospores at 20°C and then after cooling was passed over a young colony at 10°C the latter produced zygospores in the path of stream of air. On basis of this experiment they concluded that a volatile substance produced by a mature colony was able to overcome the inhibitory effect of low temperature on the initiation and early development of zygospores. Hepden and Folkes (1960) interpreted this as a provision of a methyl donor essential for the synthesis of DNA. Differential staining of vegetative and conjugating hyphae supports this view and shows that vegetative hyphal tips are rich in RNA, which in the zygospores largely disappears with corresponding increase in DNA. It is suggested that low temperature affects the nucleic acid synthesis, which in turn inhibits reproduction.

Another example of the effect of temperature on the development of perfect reproductive bodies is elaborated by *Neurospora crassa*. In this heterothallic fungus, perithecial initials are produced on monosporous colonies, but they mature only if they come into contact with a colony of conidium from a complementary strain. If after fertilization, the colony is transferred from 25°C to 35°C, the perithecial initials do not develop. Colonies grown continuously at 35°C also do not produce perithecia, whereas those grown at 35°C and subsequently transferred to 25°C produce perithecia. The results clearly reflect that temperature mature required for production of perithecial initials is higher than the temperature needed for maturation of perithecia.

As has been mentioned earlier, the temperature also influences the type of reproduction as well as the nature of spore in a particular fungus. Such phenomenon is clearly elaborated in wheat rust fungus, *Puccinia graminis tritici*. The change of uredosorus to teleutosorus is greatly influenced by temperature. Similarly, the time of development of perithecia in powdery mildews is also influenced by temperature. In Aspergilli also cleistothecia are produced at a com-



paratively higher temperature than the conidia. Temperature also influences the proliferation of sterigmata and enlargement of conidia in *Aspergillus repens* and *A. ethinulatus* (Thielke and Paravicini, 1962). Configuration as well as the dimension of the reproductive bodies in many fungi is also influenced by temperature. Sporangia of *Choanephora cucurbitarum* (Barnett and Lilly, 1950) increase in size with the increase in temperature, within a certain limit.

Production of reproductive bodies in nature is a very complex phenomenon and it actually depends on the various metabolic processes of the organism. The metabolism in turn depends on a number of factors, including temperature. Interaction with other external factors may mask or alter the nature of direct effect of temperature. Hawker (1947) demonstrated that temperature optimal for formation of perithecia of *Sordaria fimicola* is higher on a sucrose medium than on a glucose medium. In nature, however, the effect of temperature can seldom be separated from those of other environmental factors.

### Light

Visible light is known to influence sporulation and the development of spore-bearing structures in some fungi, though majority of them are not apparently affected, and they sporulate equally well in light, in darkness as well as in intermittent light. Some fungi have, however, an absolute requirement for light in order to sporulate. Quite interesting work in this connection has been carried out on *Pilobolus* by Page (1956). He suggested that some chemical substances pile up in the trophocysts of this fungus in dark. In visible light these substances get sensitized and they initiate the development of sporangiophores. Once initiated, such sporangiophores require thiamine or thiazole for further elongation. *Choanephora cucurbitarum*, *Pyronema omphalodes*, *Coprinus* spp. and certain species of *Fusarium* require short or long effect of visible light for spore-production. Often in laboratories, we observe that fungi growing in Petri-plates exhibit alternating zones of sporing and non-sporing hyphae, presenting the appearance of concentric rings. These two types of zones are actually created due to variation in frequency of sporulation in light and darkness. Such zonation rhythms under the stimulus of different photoperiods are exhibited by numerous fungi including some species of *Aspergillus*, *Penicillium* and *Irichoderma*. Formation of such concentric rings is common in many leaf-spot diseases caused by species of *Alternaria* and *Phyllosticta*. These are obviously

due to diurnal periodicity of light and darkness. Action of light in the formation of zonations may sometimes be due to its inhibitory effect upon sporulation, but generally fluctuating light excites the vegetative hyphal growth, which favours sporulation (Jerebzooff, 1965). It has been suggested that a dark period of at least 1-12 hours may be essential for formation of zonations. Alternating light and darkness are essential for spore production of certain fungi. Barnett and Lilly (1950) reported that a strain of *Choanephora cucurbitarum* produced conidia in alternating light and darkness, but sporangia were formed independently of the conditions of illumination. They concluded that at least two metabolic reactions (A and B) are influenced by light. Reaction A needs light, but strong light inhibits reaction B. Thus, in continuous bright light only reaction A is accomplished while reaction B is inhibited. In continuous darkness, however, reaction A is inhibited, and both the reactions can occur only in intermittent light and darkness. Similar two step processes appear to be involved in the sporulation of *Stemphylium botryosum* (Leach, 1968), *Thamnidium* (Lythgoe, 1961, 1962) and *Pilobolus* (Page, 1956). In *S. botryosum*, the first step appears to be the inductive phase, during which conidiophore formation is induced by ultraviolet radiation. However, in the second phase, light seems to inhibit the formation of conidia and, therefore, a dark period is essential for spore production. Light seems to be necessary for initiation of primordia among the Basidiomycetes also, or it may affect stipe elongation, pileus formation, or hymenium and spore development (Burnett, 1968).

On basis of scattered literature available on the subject the fungi may be divided into five groups with regard to their reproductive response to light: (i) those, which are apparently indifferent to light, (ii) those, in which sporulation is decreased or prevented on exposure to light, (iii) those which require alternating light and darkness to sporulate, (iv) those, which are able to produce viable spores in complete darkness, but sporulate more abundantly when exposed to light, and (v) those, which require light in order to produce reproductive bodies.

Light may affect sexual and asexual types of reproduction in entirely different manner. Its opposite effect upon sexual and asexual behaviour of *Phytophthora* has led Lilly (1966) as well as Brasier (1969) to believe that there is competition between the pathways leading to the production of the two types of spores.

The effect of different wavelengths of light on fungal reproduction has, however, been studied only in a few fungi. In general it is reported that blue-light is most effective for inducing sporulation whereas green, yellow and red may be either important (Callem-aerts, 1911; Munk, 1912), weakly active (Isaac and Abraham, 1959; Cruickshank, 1963) inactive (Hedgecock, 1906; Sagromsky, 1956), or of doubtful significance. Leach (1962, 1963) reported that doses of light needed to induce pycnidial development in *Ascochyta pisi* and *Pleospora herbarum* decreased with decreasing wavelength. Moore-Landecker (1972) has, however, mentioned that those wavelengths of light, which are either inductive or inhibitory occur in the same part of the spectrum. These are in the violet, blue, blue-green near ultra-violet or ultra-violet regions. Ultra-violet light in sublethal doses is known to promote sporulation in many fungi, like *Fusarium oxysporum* (Carlile, 1956), *Helminthosporium oryzae* (Leach, 1961) and *Alternaria dianthi* (Joly, 1962).

From the foregoing discussion, it may be inferred that the complex effect of irradiation on the physiology and biochemistry of sporulation are but little understood. Recent investigations on this aspect have progressed along three different lines, viz. (i) isolation and identification of such photoreceptor pigments, which may be involved in the photochemical reactions, (ii) determination of the action spectrum of the light induced reaction, and (iii) use of metabolic inhibitors of the pigments, and study of their effect on photo-induction of the reaction. Generally carotenoids and flavins have been considered as the possible photo-inducers, but for such a role the carotenoids need to be associated with proteins or lipoproteins, which has not yet been established (Cochrane, 1967). According to Carlile (1965), carotenoids may not be the principal photoreceptors in fungi, rather they might protect them from light-damage. From light-stimulated conidiophores of *Aspergillus giganteus*, Trinci and Banbury (1969) were able to isolate two unidentified carotenoids (besides  $\beta$ -carotene) and possibly an anthraquinone. However, none of the three could be assigned the role of photo-induction.

Leach (1962) studied the effect of irradiation on sporulation of different species of fungi, and observed that in 34 fungal species, sporulation was effectively induced by near ultra-violet radiation and was better than that by longer wavelengths, and even long exposures did not prove lethal or inhibitory. Leach (1964) suggested that in many such fungi, in which light induces sporulation, a photo-recep-

tor pigment is involved, which captures the radiant energy, particularly the ultraviolet radiation most efficiently. That the captured ultraviolet radiation influences sporulation is obvious, but our understanding of its mechanism of action leading to induction of sporulation has only made a beginning and much is yet to be learnt. However, it is now generally believed that the ultra-violet radiation exerts its influence through nucleic acid, and most probably through DNA (Moseley, 1968). However, the compounds acting as photo-receptors in fungi are yet to be identified. Leach (1965) isolated an unidentified substance (designated as P 310) with sporogenic activity from ultra-violet-treated mycelium, which resembled in its absorption curve with a thymine dimer, pyrimidine-2 dimers and an oxidation product of zeatin, although it differed from them in other physical and chemical properties. Trione and Leach (1969) consider that P 310 may be similar to these compounds, but possesses different substituent groups, and they are of the opinion that such types of photo-receptor may be functional in fungi, because according to present evidences the carotenoids, flavoprotein or pteridines do not seem to function as photo-receptors.

The biochemical aspect of photo-induced sporulation in fungi has been studied well by Cantino in *Blastocladiella*. The biochemical changes associated with the development of ordinary colourless (OC) or resistant sporangia (RS) of *B. emersonii* have been studied in detail (Cantino, 1966), which has been discussed later. While light enhances the development of OC cell during early stages, affecting a multitude of events, like nuclear reproduction, the rate of glycine uptake etc. Turian (1966) found that exogenous thymine can substitute for light in the growth of *B. emersonii*. Another species, *B. britannica* also develops into OC sporangia in light and RS in dark. It is believed that such morphogenetic response of the fungus to light is determined by the light sensitive glucose-uptake, (Horenstein and Cantino, 1964), and inhibition of light inhibits the pathway for glucose degradation, and diverts the metabolic activity towards the synthesis of polysaccharides.

### Hydrogen-ion-concentration

Extensive studies have been made dealing with the effect of hydrogen-ion-concentration on reproduction of fungi. Most of the data, however, cannot be used to reach any decisive conclusion, as they deal only with the initial pH of the medium ignoring the fact that

pH of the substrate keeps on changing due to activity of the organism. In general, a pH ranging from 5.0 to 5.8 is the most suitable for production of reproductive bodies, although quite a good number of fungi are able to reproduce from pH 3.5 to pH 8.5. Some fungi, however, require different pH optima for producing different types of reproductive bodies, for example *Mycosphaerella pinodes* has optimum pH for production of pycnidia, pseudothecia and chlamydospores at pH 5, 7 and 8 respectively (Sorgel, 1953). pH-ranges for sporulation of a few fungi have been shown in Table 14.1.

TABLE 14.1  
SHOWING pH-RANGES AND pH-OPTIMA FOR SPORULATION  
OF SOME FUNGI

Fungi	pH range for sporulation	Optimum pH range	Author (s)
<i>Absidia orchidis</i>	4.5—10.0	6.5— 8.5	} Sarbhoy (1965)
<i>Absidia</i> sp.	5.0—10.0	6.5— 8.5	
I. <i>Actinomucor elegans</i>	4.5—10.0	6.0— 8.5	
<i>Cunninghamella bertholletiae</i>	4.5—10.0	6.5— 8.5	
<i>Chaetocladium hesseltinii</i>	4.5—10.0	5.5— 7.5	
<i>Mortierella indica</i>	5.0—10.0	6.5— 8.5	} Hasija (1970)
II. <i>Curvularia pallescens</i>	2.7— 8.0	4.4— 5.4	
III. <i>C. penniseti</i>	2.8—10.0	4.4— 7.8	Agarwal (1958)
IV. <i>Helminthosporium rostratum</i>	5.6—11.2	8.1—10.5	Agarwal and Shinkhede (1959)
V. <i>H. rostratum</i>	7.0— 9.5	9.5	} Kapoor and Tandon (1967)
<i>Deightonella torulosa</i>	6.2— 8.8	8.8	
VI. <i>Botryodiplodia theobromae</i>			
Isolate I	3.5— 9.5	5.0— 6.5	} Srivastava and Tandon (1970)
Isolate II	3.5— 8.5	5.0— 6.5	
Isolate III	4.5— 8.5	5.5— 6.5	
VII. <i>Sclerotium rolfsii</i>	3.0— 8.0	5.0— 6.0	Misra and Haque (1962)

### Nutrients

Fungi come across diverse types of nutrients in nature. The capacity of majority of the moulds of being able to reproduce well in nature reflects their ability to complete their metabolic processes under varying environmental conditions. The optimum conditions under which fungi sporulate well are sometimes quite different from those required for the growth. It has been observed that sporulation of plant

parasites in nature is generally more intense, when there is a decrease in the supply of nutrients. This is, however, not universally true for all groups of fungi, as some fungal organisms may require rich quantity of nutrients to reproduce. The reasons for inhibition of reproduction by high nutrient level are not understood. It is generally believed that actual cessation of vegetative growth may be the real stimulus for initiating reproduction. In laboratories, therefore, weak media are generally recommended to induce sporulation. Inhibition of reproduction at higher concentration of nutrients may also be attributed to the excretion of metabolites by the fungus, which causes "staling" of the medium by accumulating and causing pH-changes. Presumably this staling inhibits reproduction without inhibiting the vegetative growth, because it has been observed that fungi are more sensitive with respect to their reproduction than for growth. The nature of the substrate plays a very significant role in reproduction of fungi. Laboratory experiments carried out on a wide range of fungal organisms show that concentration of the medium as well as nature of the sources of carbon and nitrogen, vitamins and mineral elements available to the fungus play a major role in determining the frequency of their sporulation. The influence of some of the nutritional sources on fungal sporulation is discussed below.

### Carbon Source

Various data collected on this aspect show that both nature as well as concentration of the carbon sources exert a decisive effect on fungal sporulation. Hawker (1939) on the basis of extensive studies on *Melanconspora destruens* reported that the perithecial concentration of this fungus declined when the amount of glucose in the medium was raised above 0.5% though the vegetative growth continued to increase even up to 10% concentration of that sugar. In contrast, the perithecial frequency on a sucrose containing medium continued to increase up to 10% concentration of the sugar. The results are interesting because glucose is one of the components liberated in sucrose hydrolysis. Decline and suppression of reproductive body formation at higher concentration of carbon sources, which generally continue to support good vegetative growth has been reported in a number of fungi including those of Phycomycetes (Sost, 1955), Ascomycetes (Hawker and Choudhary, 1946; Hawker, 1947; Buston and Basu, 1948) and Deuteromycetes (Brown, 1925; Brian and Hemming, 1950). The exact mechanism as to how high doses of carbon inhibit the sporu-



lation is not understood. It is quite likely that luxuriant vegetative growth results in greater accumulation of toxic metabolite(s) which have an adverse effect on reproduction. It has been observed that sporulation is suppressed in closed containers, which has been ascribed to accumulation of carbon-dioxide. Robinson (1926) observed that *Pyronema confluens* did not fruit in a closed tube, but in presence of an alkali which could absorb the accumulating carbon dioxide, the fungus developed perithecia. Accumulation of carbon dioxide inhibits the formation of reproductive bodies in *Chcanephora cucurbitarum* (Barnett and Lilly, 1955), *Agaricus campestris*, (Lambert, 1933) and *Collybia velutipes* (Plunkett, 1956).

In general, it has been noted that oligosaccharides and polysaccharides induce better sporulation than the hexoses. Such results can be attributed to comparatively slower growth rate on the complex carbohydrates than on the simpler ones, which support very luxuriant growth. It is also a well established fact that a large number of fungi are able to synthesize oligosaccharides of various chain-lengths when cultivated on complex carbohydrates, which in turn are again disintegrated gradually. Such activity results in a steady maintenance of the fungi at a comparatively lower concentration of the substrate. Comparatively better sporulation on complex carbohydrates, therefore, may be attributed to slower vegetative growth. Kumar and Grover (1967 a) made interesting observation with *Lophotrichus ampullus* and noted that there was no perithecial development on any of the monosaccharides, whereas oligosaccharides like lactose, sucrose and raffinose induced varying concentrations of perithecial production. The conidial production, however, does not always show such an inverse relation between growth and sporulation, because a large number of fungi, which attain good growth on glucose and other hexoses produce excellent sporulation also on those substances. Such examples are illustrated by *Aspergillus flavus* (Grover and Bansal, 1969 a), *Alternaria tenuis* and *A. citri* (Hasija, 1970), *Myrothecium roridum* (Chauhan and Suryanarayana, 1970), *Curvularia ovoides* and *C. lunata* (Singh and Tandon, 1970) *Colletotrichum gloeosporioides* (Tandon and Verma, 1962; Tandon, 1965), *C. inamdarii* (Hasija, 1964) *Pestalotiopsis versicolor* (Tandon and Mitra, 1963), *Helminthosporium oryzae* (Misra and Mukherjee, 1962) etc.

### Nitrogen

Fungi meet nitrogen requirements from nitrates, ammonium

sources, and organic sources specially the amino acids. Data dealing with the effect of nitrogen sources on fungal sporulation are rather scanty and scattered, but in general, it has been found that unlike the carbon sources, the nitrogen sources are good for growth and reproduction both. Nitrate nitrogen which is generally good for fungal growth is also reported to be good for sporulation in several fungi (Tandon and Grewal, 1956; Tandon and Bilgrami, 1957; Misra and Mahmood, 1960; Tandon and Chandra, 1961). The concentration of the nitrogenous substances required for inducing good sporulation is generally the same as is needed for attainment of good growth. High concentrations of nitrogenous substances are usually harmful for reproduction. The range of concentration supporting good sporulation is usually much narrower in case of nitrogen sources than in carbon compounds. Besides accumulation of toxic metabolites, the pH-changes in the medium due to its nitrogen content are much more sharp than due to carbon sources, and this may also be a reason for limiting good sporulation to a narrow range of nitrogen concentration. Moreover, contamination of the organic nitrogen sources with growth factors also creates complication in reaching some definite conclusions.

Asparagine, which is a good source for growth, is generally unfavourable for sporulation (Plunkett, 1953; Aschan, 1954; Agarwal and Ganguli, 1960; Dayal and Ram, 1968; Singh and Tandon, 1970). Grover (1964) reported that amino acids, which promote good growth of *Aspergillus flavus* may not enhance the sporulation in the same ratio. Presence of DL-aspartic acid, L-glutamic acid or L-lysine inhibited spore-development. Combination of two or more amino acids did not have any material effect on sporulation of this fungus.

Ammonium compounds inhibit the spore-development in *Pestalotiopsis versicolor* (Agarwal and Ganguli, 1960) and *Phyllosticta arto-carpina* (Tandon and Bilgrami, 1957). For other fungi also they are no good sources for sporulation. Singh and Tandon (1970) carried out extensive work on several isolates of *Alternaria tenuis* and concluded that there was a considerable variation in response to both growth and sporulation within the species on different sources of nitrogen but no definite conclusions regarding the relationship between growth and sporulation could be drawn.

#### Carbon-Nitrogen Ratio

Usually lower concentration of C/N ratio are recommended for



inducing and promoting fungal sporulation under laboratory conditions. Such observations have been recorded for *Botrytis cinerea* (Peiris, 1947), which exhibited better conidial production, when glucose-peptone ratio was low, while for sclerotial production the fungus needed a higher C/N ratio. Hasija (1970) recorded maximum sporulation of *Curvularia pallescens*, when the ratio of glucose and potassium nitrate was 6 : 1 (12 g C/2 gN). In *Alternaria citri* and *A. tenuis* (Hasija, 1970), the best conidial production was recorded, when the C/N ratio was approximately 4 : 1 (2 gC/0.485 gN). In certain cases, however, the C/N ratio for best conidial production expresses extreme diversities. Some of the cases showing their optimum range are enumerated here. *Colletotrichum gloeosporioides*, C/N ratio 1 : 1 to 2 : 1 (Tandon and Verma, 1962); *C. capsici*, C/N ratio 4 : 1 (Misra and Mahmood, 1960); *Fusarium coeruleum*, C/N ratio 8 : 1 (Agarwal, 1958); *Curvularia penniseti*, C/N ratio 16 : 1 (Agarwal, 1958 a).

### Mineral Elements

Studies on the effect of mineral elements on sporulation in fungi have been more or less confined to a few species only. The other main handicap in such studies has been the visual estimation of sporulation. Whatsoever the available information suggests that generally all those elements which are required for vegetative growth are also essential for sporulation. Studies on a few fungi have shown that sporulation requires more of a mineral than the minimum required for vegetative growth. *Aspergillus niger* shows drastic decline in sporulation due to a copper-deficiency, which had practically no effect upon dry-weight (Metz, 1930; Wolff and Emmerie, 1930; Mulder, 1948; Steinberg, 1935, 1936). Same kind of response was noted in *Penicillium* spp. (Bhattacharya and Basu, 1962) and *Phoma betae* (Metz, 1930). Partial deficiency of iron (Roberg, 1928) and manganese (Steinberg, 1935, 1936; Hofmann *et al.*, 1950; Bertrand and Wolf, 1955), or even potassium (Molliard 1920) and magnesium (Rabinowtiz-Sereni, 1933) also causes strong inhibition of sporulation without affecting vegetative growth. However, many fungi fail to sporulate at concentration of some minerals like zinc, well below their minimum level required for vegetative growth. This has raised the possibility that some additional mineral elements might also be playing their role in sporulation at concentration too low to be removed by the normally employed purification methods. One such additional element, which influences sporulation:

without affecting vegetative growth has been found in calcium. Basu (1951) reported that *Chaetomium globosum* growing well in a synthetic medium did not produce perithecia, unless a small quantity of calcium was added to the medium. Similar response to calcium was noted by Hadley and Harrold (1958) in *Penicillium notatum* growing in a submerged culture. However, many fungi including some other species of *Chaetomium* also show response to calcium with regard to their vegetative growth (Cochrane, 1958). Besides, calcium has been regarded more as an antagonist than as a nutrient. Advancing these arguments, Cochrane (1958) does not consider calcium as an exception to the principle that sporulation and growth do not show any qualitative difference in their mineral requirements, until more careful studies with a broader spectrum of fungal species suggest to the contrary.

### Vitamins

A number of vitamins are known to have stimulatory effect on reproduction of fungi (Lilly and Barnett, 1951; Cochrane 1958). It is, however, sometimes difficult to demarcate their influence on reproduction from that on growth. Two generalizations have, however, been accepted by most of the mycophysiologists in this connection: (i) fruiting may require more of a vitamin than a measurable growth, (ii) vitamins may accelerate sugar utilization and thereby hasten the onset of reproduction. Literature dealing with specific effect of vitamins on fungal reproduction is rather meagre. Barnett and Lilly (1947) reported that *Ceratostomella fimbriata* begins to fruit rapidly when transferred from thiamine-deficient to thiamine-containing culture solution. Relatively high doses of inositol added to a culture medium cause *Ophiostoma multiannulatum* to grow almost entirely in the form of conidia (Fries, 1949). *Sordaria fimicola* (Barnett and Lilly, 1947 a) either does not produce ascospores or produces only abortive ascospores in absence of biotin.

### ASEXUAL REPRODUCTION

Efforts have been made in the recent past to investigate the biochemical changes during the asexual and sexual reproduction in fungi. Most of the work dealing with the biochemistry of asexual reproduction concerns *Blastocladiella*, *Neurospora*, and *Aspergillus*. Some aspects have also been investigated in *Mucor*, *Saccharomyces*, *Penicillium* and *Trichophyton* etc.

Majority of the studies in *Blastocladiella* deal with *B. emersonii*, and the most extensive work has been carried out by Cantino and his co-workers. In *B. emersonii* practically the entire thallus is used up for the development of sporangium. The motile zoospore settles down, retracts up flagellum and after an exponential phase of increase in dry-weight, volume and other features, the process of cell-differentiation is initiated. The protoplasm gets segmented into a large number of spores and the structure is then referred as sporangium. The spores derived from this sporangium give rise to four different phenotypes: about 99% of the population of spores form either ordinary colourless sporangia (OC) or thick-walled pigmented resistant sporangia (RS), depending on the absence or presence of bicarbonate; about 0.5% of spores form orange coloured cells due to the presence of  $\gamma$  carotene and another 0-0.5% population comprise "late colourless cells" which differ from OC cells by their much longer generation time. Cantino and his co-workers concentrated their studies on RS and OC cells. The fact that the presence of bicarbonate during exponential phase of growth led to the development of RS sporangia provided them with an excellent opportunity to study the chain of biochemical events, where the "trigger" action leading to morphological changes depended on one single substance *i.e.* bicarbonate. Earlier work (Cantino, 1953, 1959; Cantino and Hyatt, 1953 a, b) had illustrated that OC cells are principally homo-fermentor, producing lactic acid. Cell-free preparations from OC cells exhibit most of the enzymic activities associated with the glycolytic pathway leading from hexose phosphate through exclusively NADP-specific reactions, to pyruvic and lactic acids. However, enzymic and chemical assays show that the tricarboxylic acid cycle is at least potentially operative in OC cells of various ages, although it is conceded that it is a weakly functional system playing only a minor role in supplying energy. Addition of bicarbonate to the developing germling results in quick induction of a set of enzymic lesions in the TCA cycle. Although, isocitrate dehydrogenase specific for NADP remains functional but it begins to operate in reverse direction resulting in reductive carboxylation of  $\alpha$ -ketoglutarate to isocitrate. Simultaneously bicarbonate also induces the formation of isocitrate lyase, which causes the cleavage of the isocitrate into glyoxalate and succinate and thus prevents isocitrate-accumulation. According to McCurdy and Cantino (1960), a constitutive glycinealanine transaminase finally brings about the amination of glyoxalate to glycine at the expense of alanine.

As the germling starts developing along the RS path, due to bicarbonate, its respiration drops, exogenous glucose consumption stops, and its exponential growth-rate is reduced by almost half. At enzyme level, there is an immediate exponential synthesis of isocitrate lyase. By the end of its exponential growth, the oxygen consumption of RS cells decrease to one-tenth of its level in spores, and the total intracellular accumulation of  $\alpha$ -ketoglutarate dehydrogenase shows a 90% decrease relative to the isocitrate dehydrogenase (Cantino, 1967). Such a marked reduction in the synthesis of  $\alpha$ -ketoglutarate dehydrogenase causes almost a bottleneck in the TCA cycle, which ultimately ceases to operate. However, the bicarbonate induced synthesis of isocitrate lyase comes to the rescue and immediately mediates the removal of isocitrate along the glyoxalate shunt. In OC cells (growing in absence of bicarbonate), the total activities of isocitrate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase are almost the same during the exponential phase of growth and the TCA cycle is operative uninterrupted. Moreover, there is no synthesis of isocitrate lyase, rather whatever little amount of this enzyme remains present in the spores, gets diluted out. However, the supply-route of  $\text{NADPH}_2$  and  $\alpha$ -ketoglutarate necessary for the continuation of such a metabolic route in RS development are not yet fully substantiated. Yet, it is possible that the rapid exponential synthesis of glucose-6-phosphate dehydrogenase may generate the needed  $\text{NADPH}_2$  (Cantino and Lovett, 1964), and it may be presumed that an important source of reducing power lies in the early stages of hexose monophosphate shunt. Evidences also indicate that the pathway of melanin synthesis provide a second major source of  $\text{NADPH}_2$  (Cantino *et al.*, 1957; Cantino and Horenstein, 1955, 1959; Cantino and Lovett, 1964).

When the ontogeny reaches a particular stage, the cell becomes destined to RS formation. This is referred to as the morphogenetic point of no return, and it covers 43% of the generation time in the conditions used. This point is marked with a halt to increase in cell-size and completion of cross-wall formation. Removal of bicarbonate from the medium before this point of no return, causes the reversal of the ontogeny to OC path, but beyond this point, bicarbonate's presence or absence has nothing to do with the nature of the cell. If bicarbonate is removed before the point of no return, only some of the RS characteristics revert, *e.g.* the two key enzyme systems, considered to be directly involved in RS path do revert. The total units per cell of isocitrate dehydrogenase dropped sharply,

whereas the total units per cell of  $\alpha$ -ketoglutarate dehydrogenase rose considerably (Cantino, 1961; Lovett and Cantino, 1961; Cantino and Goldstein, 1962). The events leading to the formation of RS cells many of which have been worked out (Cantino, 1967) in detail, are shown in Fig. 14.1.

The biochemistry of the final stages of development of the OC and RS cells have also been traced to a considerable extent. In final stages of RS path, total synthesis of protein and DNA stops, exogenous glucose utilization is checked, oxygen uptake and release of lactic acid into the medium decrease; but there is several times increase in chitin, melanin, lipid and polysaccharide synthesis. RS maturation is also marked with transformations in the RNA pool. There is an increase in the sodium chloride-insoluble RNA and it is believed that this insoluble RNA gets deposited in the form of protein-bound organelles. Cantino (1961) considers that these organelles are finally converted to the characteristic blastocladeaceous nuclear caps in the spores. Energy required for the synthesis of insoluble RNA is derived from the polysaccharide pool and glucose 6-phosphate dehydrogenase, because this is the only enzyme whose specific activity has been found to increase during RS maturation (Lovett and Cantino, 1961). During OC cell maturation on the other hand very little RNA synthesis takes place, and in fact, quite a considerable amount of sporangial RNA is degraded. The nuclear cap in this case does not form from newly synthesized RNA but from preexisting ribosomes (Murphy and Lovett, 1966). Further work on changes during sporogenesis, transformation of a swimming zoospore into a rounded cyst etc. has been done both at structural (Fuller, 1966; Reichle and Fuller, 1967; Lessie and Lovett, 1968) as well as functional (Cantino 1968; *et al.*, Cantino, 1969) levels, but more information will be required to establish a biochemical correlation between the two.

Work of Cantino and his co-workers is, however, not supported in totality by Khuow and McCurdy (1969). According to these workers, except  $\alpha$ -ketoglutarate dehydrogenase, all other enzymes of TCA cycle are present in the extracts from both RS and OC cells, and their per plant activity is higher in the RS cells. However, RS cells are larger than the OC cells and a comparison of specific enzyme activities showed that except aconitate hydratase and citrate synthase the activities of other enzymes were slightly lower in RS cells. In fact Khuow and McCurdy (1969) have shown striking similarities

## PERCENTAGE GENERATION TIME, RS CELL

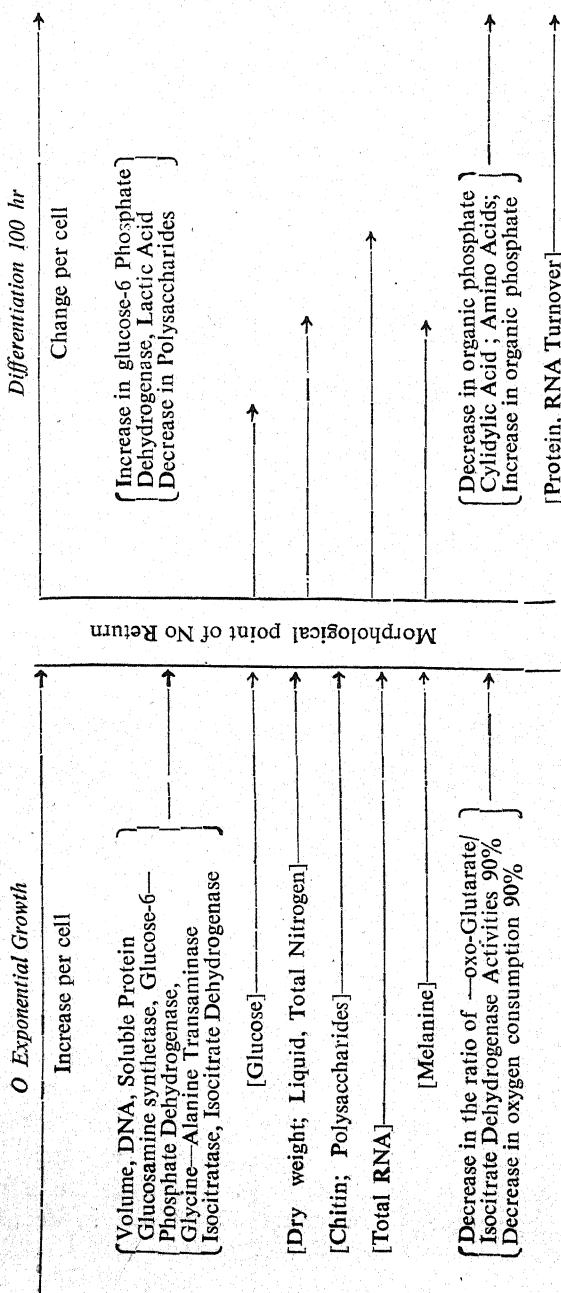


Fig. 14.1. A Diagrammatic representation of some of the events during exponential growth and differentiation of an RS cell of *Blastocladiella emersonii*.



between the TCA cycle enzyme activities (besides other characteristics) of RS and OC plants during their exponential growth. Their results contradict the earlier reports and also the very basis of Cantino's hypothesis (1965) that the TCA cycle enzymes, excepting NADP-specific isocitrate dehydrogenase, are low or lacking in the RS cells. According to Khuow and McCurdy (1969) it is only when bicarbonate has become ineffective in determining the path of development, that significant changes occur in RS development. They have made this criticism because Cantino and co-workers, have compared OC and RS cells on the basis of generation time. In their opinion (Khuow and McCurdy, 1969), the most valid comparison of RS and OC cells would be during exponential growth phase.

Further, Khuow and McCurdy (1969) used  $^{14}\text{C}$ -bicarbonate in their study and noted that the greatest amount of label appeared in aspartate, with lesser amount in glycine and malate. Significant amounts were also present in other compounds such as  $\alpha$ -ketoglutarate and lactic acid. Since aspartic acid was the first compound to be labelled and it is known to form rapidly from oxalacetic acid, it was proposed that the bicarbonate is primarily fixed at the site of  $\text{CO}_2$ -fixation either into phosphoenol pyruvate through the mechanism suggested by Stoppani *et al.* (1958), or into pyruvate through the system indicated by Woronick and Johnson (1960), to produce oxalacetic acid. The metabolism of bicarbonate would then be dependent upon a functional TCA cycle and on the relatively high levels of the corresponding enzymes existing before the point of no return. After this point, a decrease in the activities of these enzymes and a decreased rate of carbon dioxide fixation (Cantino, 1967) may be casually related to morphogenesis.

Thus, in *Blastocladiella emersonii* considerable metabolic differences have been recognised among the undifferentiated, differentiating and differentiated cells, and the evidences indicate that these differences may atleast be partially due to induction or repression of enzyme synthesis (Cantino, 1966) at one stage or the other. Knowledge of the biochemistry and physiology of differentiation in two other Blastocladales, viz. *Blastocladia* and *Allomyces*, however, could not keep pace with *B. emersonii* and lagged behind. *Blastocladia*, which is microaerophilic, indeed requires an extraordinarily high  $\text{CO}_2$  tension for production of RS cells. However the effect of  $\text{CO}_2$  on RS development has not been worked out in detail, although a

metabolic shift involving liberation of more succinate and less lactate is known to accompany the change in the morphogenetic path. (Cantino, 1966).

In *Allomyces*, the RS developmental stages are fairly well known due to notable contributions from Emerson (1950, 1955), Wilson (1952) and Rorem and Machlis (1957). However, in this fungus, attention has mainly been focussed on cytological changes during RS formation and little information is available regarding the changes in the metabolism which accompany RS formation. Cytological changes during RS path have been well described by Cantino (1966).

In filamentous fungi like *Neurospora*, *Aspergillus*, *Penicillium* etc., the study of the physiology and biochemistry of sporulation poses various problems due to their multicellular nature, different physiological conditions like oxygen tension and nutrient concentration available to submerged and aerial mycelium in a static culture etc. Even in shake or continuous cultures, uneven nutrient and oxygen availability is there, particularly when the fungus grows in the typical pellet-form (Clark, 1962). In view of these difficulties, a submerged growth apparently seems ideal, but it is never so for studies on sporulation, because proper aeration is the most potent stimulus known for conidiation. That conidiation is restricted to aerial mycelium has been shown in various filamentous fungi including *Penicillium chrysogenum* (Morton, 1961) and *Neurospora crassa* (Turian, 1969). Morton (1961) postulated that when the mycelium becomes aerial, a surface-active material, possibly protein, is formed rapidly and the dehydrogenase activity increases, showing the metabolic changes occurring in the aerial hyphae. He pointed out that the only principal difference between the aerial and submerged mycelium is the formation of an air, water interface, which changes the orientation of the polar molecules, like the unfolding of protein, which could cause metabolic changes. In *Neurospora* there is an accumulation of lipoproteins in the aerial hyphae, which causes a change in the chemical nature of the conidiophore-surface and makes them hydrophobic (Turian, 1969). Further requirement of an aerial stimulus for conidiation suggests that an oxidative metabolism is essential for sporulation, and also those conditions favouring glycolysis will inhibit conidiogenesis. When ethanol production by a vegetative mycelium was inhibited by 90-96% by the addition of p-chloromer-



curibenzoate, the mycelium initiated conidiation (Weiss and Turian, 1966). On the other hand, addition of fluoroacetate, which causes a blockage of the TCA cycle but favours glycolysis, resulted in reverting of the conidiating mycelium to the vegetative condition. Turian (1964) observed that addition of TCA cycle intermediates to a sucrose-nitrate medium greatly enhanced conidiation in *Neurospora*. This also goes to support the argument that conidiation is favoured by oxidative metabolism. Moreover, the enzymes ethanol dehydrogenase and carboxylase exhibited higher activities in the vegetative mycelium. High glycolytic activity in vegetative tissue was also confirmed by various other investigators (Kobr *et al.*, 1967; Oulevey-Matikian and Turian, 1963). Hence, Turian (1969) considered conidiation to be a morphogenetic expression of the Pasteur effect, and suggested that conidiation is regulated by the balance between oxidative and glycolytic pathways, probably at the point of pyruvate. A similar regulatory effect may also be exerted by the ratio of reduced and oxidized NAD.

It is obvious that an active hexose monophosphate pathway will favour conidiation by inhibiting glycolysis, through competition for glucose 6-phosphate. It has been suggested in *N. crassa* that the hexose-monophosphate pathway coupled to a flavin type of metabolism through NADPH<sub>2</sub>-NADP regeneration predominates during conidiogenesis. Walker and Nicholas (1962) noted that when the conidiophore takes an aerial posture and conidiates, there is a predominance of the assimilatory type of nitrate reduction requiring only the functioning of flavin adenine dinucleotide and molybdenum to regenerate NADP. Thus oxidative metabolism is although essential for conidiation, it need not necessarily be cytochrome-dependent, and nitrate assimilation through nitrate reductase may provide a mechanism for regeneration of NADP, which may be coupled with glucose 6-phosphate dehydrogenase and thus induce conidiation. McDowell and Dettertogh (1968) observed that young sporulating cultures of *Endothia parasitica* could oxidize glucose much more rapidly than comparable vegetative mycelium. There were indications to suggest that in sporulating mycelium, the hexose monophosphate pathway as well as glucuronic acid pathway operated in increased proportions.

Ammonium salts when added to the medium cause inhibition of conidiogenesis. Turian (1964) suggests that this may be due to the preferential utilization of ammonium nitrogen, which probably uncouples nitrate reductase from the hexose monophosphate pathway

and thus prevents conidial formation. Oulevey-Matikian and Turian (1969) observed that the inhibitory effect of ammonium ions may be overcome by only a single amino acid, viz. glycine, which obviously prevents the utilization of ammonium ions and permits profuse conidiation.

There have been limited attempts to elucidate the metabolic pathways crucial for conidial development. Use of appropriate metabolic inhibitors, like fluoride, arsenite, malonate, etc. has indicated that in *Aspergillus niger* conidiation requires functional glycolytic pathway as well as an operative tricarboxylic acid cycle (Behal, 1959). Galbraith (1968) concluded that in *A. niger* TCA cycle was only ticking over slowly during the rapid phase of growth due to catabolite repression. The absence of  $\alpha$ -ketoglutarate dehydrogenase activity further indicates that the TCA cycle merely serves a synthetic function, as was suggested earlier by Kornberg *et al.* (1960). In *Mucor hiemalis*, Gooday (1968 c) demonstrated that the TCA cycle was active in young stages of differentiation. He located that the TCA cycle enzymes, like malate and isocitrate dehydrogenases were most active in vegetative hyphae, zygophores, gametangia, suspensor cells and young sporangiophores, while ethanol dehydrogenase was localized to columella and chlamydospores.

In *Penicillium*, initiation of conidiation is marked by a decrease in oxygen consumption (Jicinska, 1968), but at the time of conidiophore differentiation there is a transient rise in respiration. Such a change is indicative either of a change in the respiratory substrate, or a change in the respiratory pathway from the hexose monophosphate pathway to some other oxidative metabolism, like tricarboxylic acid cycle. Deshpande and Sarje (1966) collected evidence of atleast a partially active TCA cycle during sporulation in *Penicillium frequentans*. However, the rate of conidiation may be increased markedly, by inducing the operation of glyoxylate cycle by growing the fungus in an acetate medium (Turian and Seydoux, 1961). In *Neurospora crassa*, such enhanced rate of conidiation due to a variety of environmental factors could always be correlated to higher activity of the enzyme isocitrate lyase (Turian, 1961, 1963; Turian and Kobr, 1965). The glyoxylate formed by the activity of isocitrate lyase is transaminated with alanine to form glycine (Turian and Combepine, 1963). In *A. niger* also, it is believed that isocitrate

lyase freed from catabolite repression (Polakis and Bartley, 1965), causes the cleavage of isocitrate into glyoxylate and succinate, and the former gets transaminated into glycine. The supply of isocitrate is provided by the reductive carboxylation of  $\alpha$ -ketoglutarate which accumulates due to declining glutamate dehydrogenase activity, at the time of glucose exhaustion. In *N. crassa* it is the glyoxylate and not the glyoxylate cycle as a whole, which seems to favour conidiation. Thus, glyoxylate formed either by the activity of isocitrate lyase as it happens during growth on acetate (Turian, 1961) or that produced due to splitting of the pentose during pentose monophosphate pathway (Turian, 1963) both stimulate conidiogenesis. In *Aspergillus niger*, however, Turian and Seydoux (1961) reported that acetate containing medium induced glyoxylate cycle as an alternative to TCA cycle.

Starvation, i.e., exhaustion of carbon and/or nitrogen source(s) is known to cause the initiation of conidiation in several fungi (Dicker *et al.* 1969). Galbraith and Smith (1969) observed that in *Aspergillus niger*, conidiation followed the rapid phase of growth after the exhaustion of either the carbon or nitrogen source. Further, if the carbon source (glucose) became limiting, the nature of the nitrogen source determined the initiation of conidiogenesis. With nitrate as the N-source, sporulation was induced by glucose exhaustion but ammonium salts prevented spore-induction as has earlier been indicated in the case of *N. crassa*. In both the fungi, it has been reported that the ammonium salts are able to check conidiation if added within a specified period, beyond which these salts have no effect. This shows that there occurs a critical period for spore induction in these fungi, which probably is up to 8 hrs. after inoculation in *N. crassa* (Oulevey-Matikian and Turian, 1968; Turian, 1969) and 15 hrs. after inoculation in *A. niger*. Cultures older than this do not show any response to the addition of ammonium salts. The metabolic changes caused by ammonium ions probably differ in these two fungi, because in *A. niger* their inhibitory effect is overcome by a majority of amino acids in addition to acetate, pyruvate, TCA cycle intermediates and glyoxylate. In *N. crassa*, on the other hand, glycine is the only amino acid which may overcome the inhibitory action of ammonium ions, by competing with them (Oulevey-Matikian and Turian 1968). However, the mode of action of amino acids in case of *A. niger* is not yet clear, although attempts to explain their role in terms of the activities of enzymes like glutamate de-

hydrogenase, glutamate oxaloacetate transaminase and glutamate-pyruvate transaminase have been made (Galbraith and Smith, 1969 b).

Similarly, exhaustion of nitrogen source may also induce conidiation in *Neurospora*, *Penicillium* etc. This obviously suggests that during conidiogenesis the endogenous pool of amino acid is utilized. Indeed some aconidial *Neurospora* mutants were found to sporulate on media supplemented with casamino acids. Bent and Morton (1964 a) found that conidia of *Penicillium griseofulvum* had a smaller amino acid pool than the mycelium and it showed a higher concentration of glutamine and lower level of alanine than the vegetative hyphae. Changes in the endogenous amino acid pool during growth and sporulation of a number of filamentous fungi has been followed by these workers (Bent and Morton, 1964 a, b; Bent, 1964, 1967). During rapid growth of *P. griseofulvum*, the pool mainly comprised glutamate and alanine, and till the exhaustion of nitrogen source, there was no substantial change therein. But after that there occurred a rapid depletion in the glutamate, ornithine and arginine levels, while alanine declined more gradually. Similar changes were observed in the amino acid pools of *P. chrysogenum*, *P. expansum*, *Aspergillus niger* and *Trichoderma viride*. Moreover, nitrogen exhaustion was found to induce in the vegetative mycelium of *P. griseofulvum*, a rapid break-down of insoluble nitrogen into amino acids (Bent, 1964). Later, new protein bands and changes in the intensity of the pre-existing ones, were observed on electrophorogram of vegetative mycelium growing in nitrogen depleted cultures. These observations lead to the conclusion that during sporulation initiated by nitrogen-starvation, considerable reorganisation of the nitrogenous constituents takes place. Other metabolic changes caused by nitrogen-exhaustion as well as the mechanism by which it induces conidiation are not yet fully understood. Most of such studies have been made while carbohydrate was still present in the medium rather in a high concentration. This has raised doubts whether the stimulus for conidiation is entirely provided by the exhaustion of nitrogen source, or the carbon source also plays some part. Such concept has particularly arisen from the observation of Morton *et al.* (1958) that sporulation may be induced in submerged cultures of *P. griseofulvum* by replacing the pure glucose by a "crude glucose" derived from maize starch by acid hydrolysis. Armstrong *et al.* (1963) suggested that the cause of such stimulus may be the presence of anhydro-

glucose and traces of  $\text{Ca}^{++}$  in the crude glucose. Whatever may be the cause, this much seems possible that such a crude glucose is able to induce atleast some of the metabolic changes which are also caused by exhaustion of nitrogen.

Available data suggest that in general, the metabolic activities of spore-bearing hyphae are higher than the vegetative mycelium. Comparison of sporulating and vegetative mycelia have generally been made either on the basis of concentration of metabolic products or enzymatic activities. Evidence has been collected in certain fungi, that nucleic acid contents are higher in conidia than in vegetative mycelium. Budding conidia of *Ophiostoma multiannulatum* have higher DNA content than the hyphae (Von Hofsten, 1962). Maruyama and Alexander (1962 b) found microconidia of *Fusarium oxysporum* f sp. *cubens* appreciably rich in nucleic acid. In *Neurospora crassa*, Owens *et al.* (1958) recorded an average RNA:DNA ratio of 8:7 in the conidia. However, Minagawa *et al.* (1959) did not find any difference in the base ratio of total RNA in hyphae and conidia. Turian (1969) has demonstrated the physiological differentiation of mycelial and conidial cultures of *N. crassa* on the basis of biochemical analysis. Recently, Griffin and Breuker (1969) have made an interesting correlation between RNA synthesis and sporangial development in *Achlya*. From their studies with labelled compounds, they concluded that DNA dependent RNA synthesis was necessary for sporangial differentiation, and during sporangial development, a characteristic RNA fraction was obtained, which was heavier than ribosomal RNA. Changes in RNA contents have also been studied in *Trichoderma* by Gressel and Galum (1967), but they too could not arrive at some precise inference.

In *Penicillium roquefortii* the formation of ketones during fatty-acid metabolism was found to be associated with spores and not with the mycelium (Gehrig and Knight, 1958). Recently Bianchi and Turian (1967) also concluded that lipid synthesis is connected with conidiation. In respect of nitrogen content, however, vegetative mycelium is richer than conidiating mycelium as well as conidia. Stokes and Gunness (1946) found vegetative mycelium of *Aspergillus niger* containing 50% more protein (made up of all amino acids except histidine) than the conidiating mycelium. Conidia and sporulating mycelium exhibited only about 10% difference in their nitrogen content, with the mycelium possessing about three times higher concentration of histidine than the conidia. Yangita and

Kogane (1962, 1963) obtained results which indicated that certain basophilic substances, probably nucleoproteins, were transported into the conidia through the conidiophores. Bajaj *et al.* (1954) demonstrated that certain phosphorus containing compounds were also translocated from the mycelium into the conidia, where they probably formed acid-soluble polyphosphates and accumulate as meta-chromatic granules (Kulayev and Belozersky, 1957; Nishi, 1961). These polyphosphate reserves perhaps are needed for the successive nuclear divisions occurring in the phialides. Conidia also contain sulphur-protein and good amount of other sulphur containing compounds, like choline sulphate.

Study of enzymic activities in *Neurospora* was carried out by Zalokar (1959 a, b), who found that conidiation was associated with an increase in succinate dehydrogenase activity. Later, Stine (1967) as well as Ohja and Turian (1968) also arrived at similar conclusions. The former could apparently correlate the conidiophore differentiation in *N. crassa* with enhanced activity of NAD nucleotidase and NAD-dependent glutamate dehydrogenase (Stine, 1968) and thereby confirmed the earlier concept of Stachow and Sanwal (1964) that the NAD specific glutamate dehydrogenase may be involved in glutamine synthesis, while its NADP-specific counterpart may act in normal metabolism during the log phase of germination and mycelial growth.

In *Aspergillus niger*, Nagasaki (1968 a) recorded drastic changes in the activities of ribonuclease and deoxyribonuclease on the day conidiation started and also when the conidia matured. Similarly, the activities of acid and alkaline phosphatases were also much pronounced in sporulating structures than in hyphae. Gooday (1968 c) in *Mucor hiemalis* found that the distribution of TCA cycle enzymes and ethanol dehydrogenase enzyme was complementary. While a biochemical breakthrough in the physiology of sporulation is yet to be achieved despite continuing activities, as is clear from the foregoing discussion, some entirely different possibilities of morphogenetic control mechanisms have also been suggested from time to time e.g. specific sporulation factor and extra-chromosomal particles etc.

It was noticed by Hadley and Harrold (1958 a, b) that the culture-medium obtained from a mature mycelial growth induced conidiation in *Penicillium chrysogenum* more rapidly than a fresh medium. Similar effects were observed of media, in which various *Penicillia* or *Aspergilli* were pre-grown. Such stimulatory effect of pre-grown media was interpreted as due to the presence of sporulation factor(s)



and not as an effect of staling. Sporulation-factor of this kind has since been recorded for *Fusarium*, *Geotrichum* etc. (Park, 1961, 1963; Park and Robinson, 1964, 1969; Robinson and Park, 1965). Even a volatile factor, probably ammonia (Page, 1959), has been reported to be formed by *Mucor plumbeus*, which stimulates sporulation in *Pilobolus*.

Indication for an extra-chromosomal particle controlling sporulation was obtained by Cantino and Hyatt (1953 c) as well as Cantino and Horenstein (1954). They noticed that a cytoplasmic factor, which they called as gamma particle, influenced the development of orange, late colourless, and OC plants from RS plants. In *Aspergillus glaucus*, Mather and Jinks (1958) noted a loss of reproductive capacity, when the fungus was propagated for several generations either by conidia or by hyphal tips. Later Jinks (1966) concluded that cytoplasmic systems have a definite role in fungal growth and sporulation. Bertrand *et al.* (1968) have now recorded similar cytoplasmic factor for sporulation in *Neurospora crassa*. However, these limited investigations indicate only a beginning in this particular direction, and only sustained efforts might reveal their actual mechanism of action, as well as their extent of distribution among fungi.

### SEXUAL REPRODUCTION

Like other living organisms, sexual reproduction in fungi also, involves the union of two compatible nuclei. From the sexual point of view, fungi may be classified as hermaphroditic, dioecious or sexually undifferentiated. On the basis of available literature, it can be decisively concluded that sexual reproduction in fungi is a physiological mechanism superimposed on the genetic genome, which governs sexuality. Although the role of sex-hormones (gamones or sporogens) is now well established in several fungi, yet the details of the various physiological and biochemical processes are still obscure in a majority of them. Some of the sex-hormones have been chemically characterized in the recent past and more revealing facts may come to light in due course of time.

A wealth of information has accumulated on sexual development of some fungi, like *Allomyces*, *Blastocladiella*, *Achlya*, *Mucor*, *Blake-slea*, *Saccharomyces*, *Ascobolus*, *Schizophyllum* etc. However, unless more is known about meiosis and its physiology, particularly the underlying principle which governs the transition from mitotic to



meiotic division, a fuller knowledge of the mechanism of recombination in eukaryotic organisms in general and fungi in particular, will be difficult to achieve. Sexual reproduction in fungi is also marked by meiosis, which in a majority of fungal organisms occurs as a result of some preceding events, like karyogamy or during germination of zygote. Otherwise, as in certain yeasts, the prevailing environmental conditions may also induce meiosis.

### **Heterothallism in Fungi**

As mentioned earlier, a large number of fungi express the phenomenon of heterothallism, which was first discovered among the Mucorales by Blakeslee (1904). *Rhizopus nigricans*, the common black breadmold was cited as the first case of obligatory cross-breeding in fungi. Subsequently, a large number of Mucorales were unambiguously categorized as heterothallic or homothallic. Heterothallism is a common phenomenon, which besides the zygomycetes has been found to occur in all the major groups of fungi: in the Hymenomycetes (Bensaude, 1918; Kniep, 1920, 1922); in Ustilaginales (Kniep, 1929); in Saprolegniaceae (Couch, 1926); in Euscomycetes (Shear and Dodge, 1927); in Uredinales (Craigie, 1927); in Blastocladales (Harder and Sorgel, 1938) and in slime molds (Dee, 1960). A fungus which is self-fertile, and will either fertilize itself or mate with a similar strain is *homothallic*. In contrast, a *heterothallic* fungus is one, which is self-sterile and requires a compatible partner for development of the perfect stage. According to Whitehouse (1949), there are two fundamentally different types of heterothallism, viz (i) morphological heterothallism, where morphologically distinguishable gametangia are produced by two different thalli, and cross-breeding becomes obligatory; morphological heterothallism is common in many lower fungi; and (ii) physiological heterothallism, which depends on genetic factors, which determine their compatibility and incompatibility, and male and female thalli can not be distinguished morphologically.

With reference to Mucorales, Blakeslee (1906) designated these physiologically and genetically different strains as of plus (+) and minus (−) strain. Sometimes physiological heterothallism and morphologically distinguishable sex-organs may occur on the same thallus, or morphologically distinct sex-organs may occur without an accompanying physiological compatibility factor. A fungus with

physiological heterothallism may lack differentiated sexual organs (as in Homobasidiomycetidae), or it may have morphologically distinct sexual structures. In the latter case, even if male and female organs occurred on the same thallus, the fungus would not be able to fertilize itself, as it has a basic requirement for genetically different nuclei. *Ascobolus stercorarius*, *Sordaria anserina*, some species of *Neurospora* and the rust fungi are the examples of such type. As indicated earlier, physiological heterothallism is genetically controlled and two types of genetic mechanism are known to be involved, viz. (i) two allele heterothallism and (ii) multiple allele heterothallism.

(i) *Two allele heterothallism*. In this type, two allelomorphs situated at the same locus are involved. This type of heterothallism has been recorded in members of Mucorales, like *Mucor*, *Rhizopus* and *Phycomyces*, most species of *Neurospora*; *Ascobolus magnificus* and *Sclerotinia gladioli*; the rust fungus *Puccinia graminis*; and *Ustilago levis* (White house, 1949). "In all these species two different strains are recognised, which are commonly referred as 'plus' and 'minus' or *A* and *a* strains (White house, 1949) and (+) strains can only mate with (—) strains and not with (+) strains.

(ii) *Multiple allele heterothallism*. In this kind of heterothallism, the sexual compatibility is controlled by multiple alleles, which are all situated on the same locus. This enhances the chances of compatible crossing and hence the degree of outbreeding is also increased. Two types of multiple allele heterothallism may operate:

(a) *Bipolar multiple allele heterothallism*. In this mechanism, sexuality is controlled by a multiple allelic series situated at a single locus to control sexuality (Whitehouse, 1949). In a compatible crossing of this type, the mating thalli must bear different alleles. A thallus bearing the allele 'A' of a multiple allele system,  $A_1, A_2, A_3, A_4 \dots A_n$ , can mate with a thallus containing any of the remaining alleles. (Fig. 14.2). This kind of bipolar multiple allele heterothallism has been reported in most smut fungi (Raper, 1960; Halisky, 1965), some Gasteromycetes (Fries, 1948; Burnett and Boulter, 1963), and *Coprinus comatus* (Whitehouse, 1949).

	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>	A <sub>4</sub>
A <sub>1</sub>	—	+	+	+
A <sub>2</sub>	+	—	+	+
A <sub>3</sub>	+	+	—	+
A <sub>4</sub>	+	+	+	—

Fig. 14.2. Showing mating reactions of a bipolar fungus, with four possible isolates, (+)=compatible cross  
(—)=incompatible cross.

(b) *Tetrapolar multiple allele heterothallism*. In this type of heterothallism, there are two different loci which control sexual compatibility, and at each locus there is a series of multiple alleles. These two loci (A and B) segregate independently during meiosis. According to Raper (1953) there are at least 100 alleles at each locus. In tetrapolar fungi, a fully compatible cross can only be established between thalli which differ with respect to both the loci. For instance, a thallus with A<sub>1</sub> B<sub>1</sub> alleles will make a compatible cross only with a A<sub>2</sub>B<sub>2</sub> thallus.

	A <sub>1</sub> B <sub>1</sub>	A <sub>1</sub> B <sub>2</sub>	A <sub>2</sub> B <sub>1</sub>	A <sub>2</sub> B <sub>2</sub>
A <sub>1</sub> B <sub>1</sub>	—	FL	P	+
A <sub>1</sub> B <sub>2</sub>	FL	—	+	B
A <sub>2</sub> B <sub>1</sub>	B	+	—	FL
A <sub>2</sub> B <sub>2</sub>	+	B	FL	—

Fig. 14.3. Showing mating reactions of four possible isolates of a tetrapolar fungus.

A cross between A<sub>1</sub>B<sub>1</sub> and A<sub>1</sub>B<sub>2</sub> or A<sub>2</sub>B<sub>1</sub> will not be fully compatible, obviously because the two mating thalli will have common alleles either of A series or B series. Crossing between mycelia with either A or B allele common to the two mating partners has been successful in some fungi to the extent that heterokaryons are established,

but generally no fructifications are formed. Such incomplete compatibility results in disturbed growth and morphology of the heterokaryon. Raper and San Antonio (1954) observed that the growth of a heterokaryon with common A alleles but different B alleles, is sparse, depressed and with gnarled, irregularly branched older hyphae. Such characteristic growth response is designated as flat-reaction. When the heterokaryon bears common B alleles but dissimilar A alleles the characteristic growth response is termed the barrage reaction. In such cases, the hyphae of the mating mycelia grow towards each other but near the centre their growth ceases. This results in the formation of a zone (barrage) of little growth inbetween the mating colonies (Papazian, 1950; Raper, 1953). Effect of common A and common B alleles on heterokaryon has been studied in detail by Parag (1965) in the fungus *Schizophyllum commune*. According to him, common A alleles prevent the formation of clamp-connections, disrupt the regularity of nuclear distribution, besides producing morphological and metabolic abnormalities in the mycelium. Similarly, common B alleles prevent nuclear migration, clamp-connection as well as karyogamy and interferes with meiosis.

Middleton (1964) has reported the occurrence of common AB heterokaryon in the fungus *Schizophyllum commune*, and observed that the mating mycelia over-ran each other in such crosses. Tetrapolar multiple allele controlled heterothallism has been reported in a majority of Hymenomycetes, Gasteromycetes, as well as the smut fungus *Ustilago maydis*.

Heterothallism is a fascinating problem of research and the exact mechanism which leads to the development of perfect spores in many complimentary strains of heterothallic species is yet to be discovered. As early as 1928 Gwynne-Vaughan suggested that the difference between the two strains was based on nutrition, and each strain was independently capable to synthesize certain substances or to perform some steps which were essential for fruiting. They were thus complimentary to each other. Burgeff (1924) carried out extensive work with heterothallic colonies of *Mucor mucedo* and he observed that when the colonies of (+) and (-) strains approached one another there developed a zone of resistance between them, which was penetrated only by a few hyphae from each colony. The tips of these hyphae develop irregular swellings, before they came into actual contact. Such behaviour obviously suggests that some diffusible or volatile substance(s) produced by a particular strain influences the

behaviour of the opposite strain, in advance of actual physical contact. This was the first demonstration of a hormonal sexual mechanism in the fungi. Burgeff's observations were subsequently confirmed by Kohler (1935), who concluded that both the mating strains produced diffusible substances. However, demonstration of such chemical agents in the culture-medium of mated strains of *M. mucedo*, which could induce zygomorph production in unmated cultures of the same fungus, could only be possible after two decades (Burgeff and Plempel, 1956). Krafczyk (1935) demonstrated a mutual attraction of hyphae of opposite strains and initiation of gametangial initials in *Pilobolus crystallinus*. He concluded that initiation as well as subsequent development of sexual reaction is controlled by hormones upto the stage at which gametangia are delimited. Burnett (1953) carried out interesting experiments with *Mucor hiemalis* and *Phycomyces blakesleeanus*. He recorded a significant increase in oxygen consumption just before the two approaching colonies of opposite strains came in actual contact. Banbury (1954, 55) collected more evidences regarding the diffusible nature of the stimulus. He noted that zygomorph formation was accomplished, when the liquid which had previously passed through a (+) and then a (-) culture reached a second (+) culture in a series of such alternating cultures, maintained in microaquaria. It was thus concluded that the stimulus inducing zygomorph formation was not air-borne. Banbury elaborated his experiments further and concluded that mutual attraction of the zygomorphs of opposite strains could possibly depend on some air borne stimulus, provided the gap between two zygomorphs of opposite strains was as narrow as 1 mm. Perhaps this is the first record to show that stimulus causing the formation of zygomorphs was different from the stimulus needed for subsequent elongation and directional growth of the zygomorphs. Banbury's work also supports Krafczyk's hypothesis that conjugation in the Mucorales is controlled by hormones during earlier stages. These substances are considered to be of highly labile type and they are possibly destroyed by oxidation on separation from the living mycelium.

However this did not discourage efforts for their isolation and characterization. Plempel (1963 a, b) extended his earlier findings and concluded that in unmated cultures both the strains of *M. mucedo* produced separate hormonal substances (progamones), which then induced the respective opposite strain to form another hormone, again by both the mating types, and thus two kinds of gamones were

found in the mated culture. Isolation-efforts have also met with success in the recent past. Gooday (1968 a, b) has isolated and characterized a hormonal substance mostly from the mycelium from mated cultures of *M. mucedo*, which could induce zygophore formation in unmated cultures of *M. mucedo*. *Blakeslea trispora*, also produces a sex-hormone, the trisporic acid (oxidized unsaturated derivative of 1, 1, 3-trimethyl 1-2-(3-methyloctyl) cyclohexane; Fig. 14.4, which shows close similarities in its biological property to the one produced by *M. mucedo* (Caglioti *et al.* 1964; Cainelli *et al.* 1967; Sutter and Rafelson, 1968). Owing to identical chemical structure, the hormone produced by one fungus is sometimes functional for another fungus. Ende (1967) obtained a substance from the mated cultures of *B. trispora*, which was active for *Mucor mucedo* also. In fact, active substances obtained from a number of Mucorales exhibit close affinities to trisporic acid B and C (Ende, 1968; Austin *et al.* 1969 b).

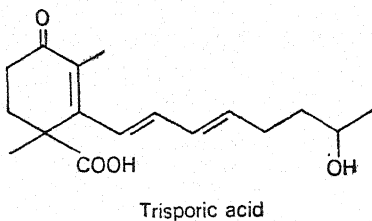


Fig. 14.4. Structure of trisporic acid.

Hormonal substances are also known to induce the development of male and female reproductive bodies in Blastocladales, Saprolegniales and Peronosporales. As early as 1881, deBary reported that certain chemical substance emanating from the oogonia of *Saprolegnia*, *Achlya*, *Pythium* and *Phytophthora* initiated the development of the antheridial hyphae and they directed the growth of these hyphae towards the oogonia. Kauffman (1908) also confirmed deBary's observation with *Saprolegnia hypogyna*. Couch (1926) demonstrated the operation of hormones in the reproduction of *Dictyuchus*. Perhaps the most elaborate and exhaustive investigation in this connection was carried out by Bishop (1940) with *Sapromyces reinschii*, who gave unequivocal proof that secretion from the female plants induced the production of antheridial hyphae on the male plants, and the

growth of the antheridial hyphae was always towards the oogonial initials. Bishop concluded that production of antheridial branches was a direct response to diffusible substances produced by the female mycelia, and thought that the initiation of the oogonia and the growth of the antheridial branches towards them were under hormonal control. Most interesting work in this connection has been carried out by Raper (1939, 1940, 1942, 1950, 1951) with two species of *Achlya*, viz. *A. ambisexualis* and *A. bisexualis*, which are morphologically heterothallic, and bear the male (antheridia) and female (oogonia) reproductive organs on two different thalli. The conclusions derived by Raper may be summarised in the following five steps:

- (1) Production of fine branched antheridial hyphae on the male plant. This stage is controlled by hormones A and  $A_2$  by the female vegetative plant. The male plant subsequently produces hormone  $A_1$ , which passes back to the female plant augmenting the continued production of hormones A and  $A_2$ . Eventually, the male plant produces hormone  $A_3$ , which depresses the action of A and  $A_2$ .

- (2) Production of oogonial initials on the female plant. This is controlled by hormone B produced on the antheridial hyphae.

- (3) Directional growth of antheridial hyphae towards the oogonial initial, and delimitation of the antheridia by formation of cross-walls behind the tips, after these tips have come into contact with the oogonial wall. This stage is controlled by hormone C, produced by the oogonial initials.

- (4) Delimitation of oogonia by the formation of basal septa and the differentiation of the protoplasm in the oogonia to give several spherical oospheres. This stage is controlled by hormone D produced by the antheridia.

- (5) The penetration of the oosphere wall by fine fertilization tubes produced by the antheridium, and the migration of the antheridial nuclei through these to the oosphere.

According to Raper (1951, 55), sexual differentiation in *Achlya* is controlled by at least seven different hormones, four of which are secreted by the male thallus and three by female thallus. All these hormones are effective even in very minute concentration. They are supposed to be water soluble and external factors like temperature, hydrogen-ion concentration and nature of the nutrient in the medium



influence their activity. Exact chemical nature of most of these hormones, however, has not been determined due to their feeble

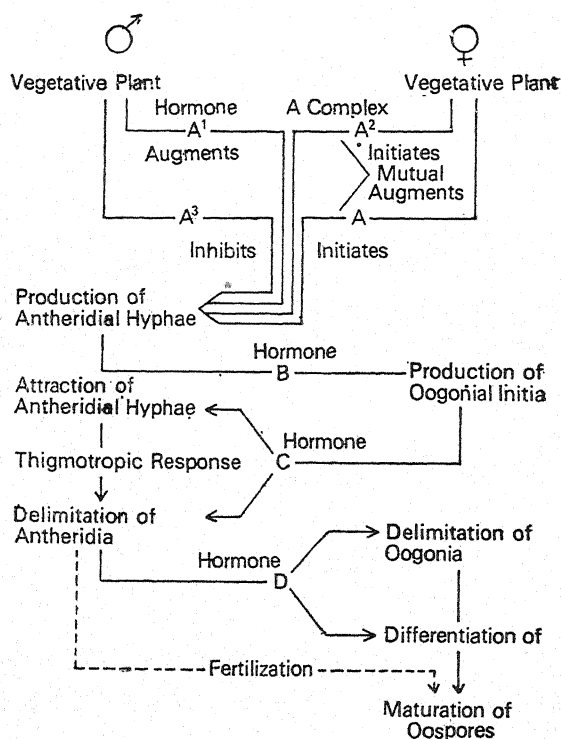
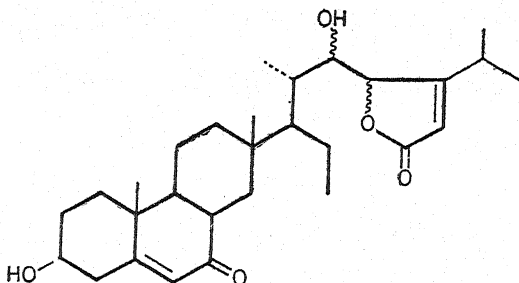


Fig. 14.5. Hormonal mechanism which co-ordinates the sexual Interactions between male and female thalli of *Achlya* spp. (after Raper 1951, 52).

concentration and highly labile nature. Raper also demonstrated that certain homothallic species of *Achlya* and species of a related genus *Thraustotheca* possess sexual mechanism, resembling that of heterothallic *Achlya* spp. Interspecific and intergeneric mating of homothallic mycelia with male and female thalli of heterothallic strains yielded all degrees of reactions from complete indifference to complete compatibility, but most often the chain of reactions failed to continue beyond a point characteristic of a particular combination of strains. This obviously suggests that hormonal substances produced by

different strains or species are similar but not identical. Recently, one of the hormones secreted by *Achlya* spp. has been isolated and characterised (McMorris and Barksdale, 1967; Arsenault *et al.* 1968). Secreted by female plant, the hormone has been designated as antheridiol, obviously on the basis of its function, regulating the formation of antheridia. The structure proposed (Fig. 14.6) for antheridiol is quite interesting and is the first steroidal sex hormone in plant kingdom, and differs from mammalian sex-hormones only in the length of its side chain.



## Antheridiol

Fig. 14.6. Structure of antheridiol.

Another water-mould, *Allomyces*, has also furnished evidence of hormonal activity at the time of sexual reproduction (Machlis 1958 a, b, c; Machlis *et al.* 1965). The gamete bearing generation in this fungus comprises dichotomously branched system of mycelium, which bears a large number of small orange coloured male gametangia and comparatively bigger and colourless female gametangia. The orange colour of the male gametangia is on account of  $\gamma$ -carotene synthesized by the fungus. Cytological investigation (Emerson and Wilson, 1954) rule out the possibility of involvement of sex-chromosomes in determining the ratio of male and female gametangia in *Allomyces* which is normally 1 : 1. Turian (1960) induced female strains to revert partially to maleness by growing them on a synthetic medium with acetate as a single source of carbon and traces of coenzyme A or with glucose as the carbon source enriched with glycine and folic acid. The same media induced a high degree of maleness (80 to 90%) in a normally bisexual strain with a 1 : 1 male and female

ratio. Such behaviour goes to support the view regarding impact of physiological conditions on morphological differentiation of sex in *Allomyces*.

In water the gametangia release highly active, motile male gametes and sluggishly motile colourless female gametes. The clustering of male gametes in the vicinity of unopened female gametangia can be clearly observed. This is brought about by a sperm-attractant hormone "sirenin" liberated from the female gametangium (Machlis 1958 a, b). It has been demonstrated that purified sirenin attracts the male gametes at concentrations as low as  $10^{-16}$ , and gamete attraction increases with the increase in its concentration upto  $10^{-6}$ . However, it fails to exert any attraction at concentration as high as  $10^{-4}$ . Carlile and Machlis (1965 a, b) found that sirenin once exposed to the male gametes lost its activity, and that the female gametes did not respond to sirenin. Sirenin has been shown to be an oxygenated sesquiterpene with four degrees of unsaturation and with both of its oxygen functions capable of forming esters (Fig. 14.7). Isolation and chemical characterization of sirenin has been described by Machlis *et al.* (1966). It is also considered that there may be several species-specific sirenins (Machlis, 1968).

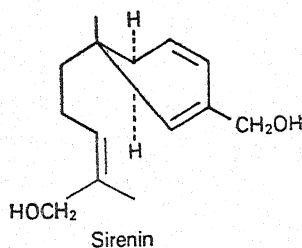


Fig. 14.7. Structure of sirenin.

It is interesting to note that carotene possibly plays an important part in sexual reproduction of these fungi, particularly those belonging to Mucorales and Blastocladales. Thomas and Goodwin (1967) reported that when a crude extract of medium of mated culture of *Blakeslea trispora* was added to a minus (—) mating type culture of that fungus, the carotene-production was enhanced. Such an increase in carotene content has been correlated with the production of zygophores and has been considered as the by-product of the action of

trisporic acid causing zygothecium formation. However, Austin *et al.* (1969 a, b) have suggested just the other way-round and they consider the hormone trisporic acid as a product of  $\beta$ -carotene metabolism. In *Phycomyces* the latter concept seems to hold good, because a mutant deficient in  $\beta$ -carotene (*car*-mutant) was also deficient in sexual-phase.

In higher Oomycetes, particularly *Pythium* and *Phytophthora*, a hormonal control of sexuality has not yet been demonstrated. Some recent investigations (Elliott *et al.* 1964; Haskins *et al.* 1964; Hendrix, 1964; Chee and Turner, 1965) have indicated the necessity of sterols in the sexual behaviour of these fungi, although they perhaps lack a complete sterol synthesizing system (Appleton *et al.* 1955; Cochrane, 1958). Obviously on a sterol-deficient medium, these fungi attain vegetative growth only (Elliott *et al.* 1964; Hendrix, 1965; Schlosser and Gottlieb, 1966). Although the role of sterols in the formation of sexual organs is little understood, it has been suggested that the stimulatory effect of sterols on sexual reproduction in *Pythium* may depend upon the ion (Lenney and Klemner, 1966) and amino acid (Sietsma and Haskins, 1967) composition of the medium. Recently, Child *et al.* (1969) recorded about three times higher number of oospore formation by cholesterol stimulated *Pythium* culture grown with asparagine as the N-source than with alanine or arginine.

The physiology and biochemistry of sexual reproduction of Euscomycetes is far less understood, although descriptive information about the development of sex-organs in these fungi abound. Moreover, there has been unequal stress over different aspect, *e.g.* the biochemical aspect of male or conidial stage of *Neurospora* has been extensively investigated by Turian and his team, while its ascogonial stage has practically been overlooked. The possibility of hormonal involvement in the sexual reproduction in these fungi was first proposed in the beginning of this century by Dodge (1912), and latter a multihormonal system was almost confirmed by Bistis (1956, 1957) in atleast one member of this group, but as yet no hormone has either been isolated or characterized. Investigations regarding involvement of hormones in perithecial development in *Neurospora* virtually led to a controversy, which has been well exposed by Raper (1952), and which ultimately proved abortive. Thus, sex-hormones have never been unequivocally demonstrated in *Neurospora* (F. Moreau and Maruzi,

1931; F. Moreau and Moreau, 1938; Aronscue, 1933; Dodge, 1935 a; Lindegren, 1936; Sansome, 1946). However studies with *Glomerella* (Markert, 1949; McGahen and Wheeler, 1951; Wheeler, 1954; Driver and Wheeler, 1955) indicated the possibility of a diffusible substance influencing the perithecial development, but it seems that the work has not been carried to its logical conclusion as yet. Possibility of hormone-control in these fungi in relation to chemotropic growth of trichogyne was indicated by Dodge (1912). However the first decisive evidence for a chemotropic growth response of trichogyne was provided by Zickler (1937, 1952) in *Bombardia lunata*. Trichogyne of this fungus first grows in a random manner, but in presence of spermatium of the right mating type, the trichogyne makes a direct growth towards it, even by making sharp bends. Zickler found the trichogyne, and not the vegetative mycelium, responding in an identical manner to films of water, in which spermatia had been soaked for some time and then removed by filtration. This water was effective even after autoclaving. Water-infusion prepared from vegetative hyphae of the correct mating type also had similar effect upon trichogynes. However, no hormonal substance has been isolated from this fungus also.

Bistis (1956, 1957), by his elaborate studies on *Ascobolus stercorarius*, has implicated a multihormonal system in influencing and regulating sexual reproduction in this fungus. He observed that the male reproductive body of this fungus, the oidium, induced ascogonial development only after the oidium itself got sexually activated by exposure to the mycelium of the opposite mating type for about 4 hrs. The activated oidium in turn stimulated the development of ascogonial initials, which ultimately gave rise to ascogonia with trichogynes. The trichogyne showed a chemotropic response towards the oidium, and changed its direction of growth as and when the oidium was shifted from one place to another. Bistis repeated such manipulations several times and found the trichogyne responsive till its growth stopped. The activation of oidia in the presence of mycelium of the right mating type has been considered as induction of antheridia by Machlis (1966) due to some hormonal action. Bistis and Raper (1963) had earlier reported that hyphal fragments may be substituted for oidia as fertilizing elements. The hyphal fragments show dilation of their tip as they become sexually activated. Machlis (1966) considers this also as antheridial induction. Bistis (1957) indicated that during sexual activation, some changes also occurred

in the oidial wall, which resulted in its breaking at the point of contact with the trichogyne wall (itself locally dissolved). These observations provide evidences for involvement of more than one hormone in sexual-reproduction of this fungus. However, none has been isolated as yet.

Other aspects, like morphogenesis of sex-organs and the metabolic pathways favouring sexual reproduction in these fungi have also received considerable attention, particularly in forms like *Neurospora*, yeasts, *Aspergilli* and *Penicillia*, *Ophiostoma*, *Glomerella*, *Venturia*, *Podospora* and *Sordaria*, yet much is still unknown.

Investigation on sexuality in *Neurospora* was initiated in *N. sitophila* (Shear and Dodge, 1927; Dodge, 1928) and was soon followed in *N. crassa* (F. Moreau and Moreau, 1930; Dodge, 1935 a; Backus 1939) and the homothallic species *N. tetrasperma* (Colson, 1934). Then there was a predominance of genetical and particularly mutational studies during which several morphological and biochemical mutants were obtained. A minimal medium for perithetical production, devised during these investigations (Westergaard and Mitchella, 1947), proved to be a good tool for the study of metabolic changes during sexual reproduction. However, they noted that the same medium did not support perithecial formation at 35°C. Hirsch (1954) not only confirmed such temperature-effect on reproduction of this fungus, but carried his observation further and noted the biochemical changes in such temperature induced sterile mycelium. He could conclude that in such sterile mycelium the enzyme tyrosinase as well as melanine were either reduced or completely lacking. In fact, he established a correlation between melanin synthesis and formation or normal functioning of protoperithecia, and suggested a causal relationship between tyrosine metabolism and initiation of female sex-organs. Hirsch's concept was supported by the observation that female-sterile strains (Barbesgaard and Wagner, 1959) also exhibited lack of tyrosinase activity. However, no direct evidence is available to suggest that during the ontogeny of female sex-organs melanin precursor(s) exert some kind of hormonal effect (Westergaard and Hirsch, 1954) although Ito (1959, 1961) found some substances present in the filtrate of a *N. crassa* strain, which could act as sex-inducers for the opposite mating type. Horowitz *et al.* (1961) considered that melanin-production actually reflected the protein turn-over during protoperithecial differentiation.

Turian and his coworkers have tried to establish metabolic distinctions between different morphogenetic stages of *N. crassa*. From the

striking accumulation of succinic dehydrogenase recorded in the conidial, "male" filtrate, they concluded that any lesion in the TCA cycle seemed to favour conidial differentiation, while a fully functional TCA cycle was required for protoperithecial development. These possibilities also got support from observations that malonate, an efficient TCA cycle inhibitor, strongly inhibited protoperithecial morphogenesis (Turian, 1962 c) in *N. crassa* as well as *N. tetrasperma*. Further, induction of glyoxylate cycle as an alternative to the TCA cycle (by providing acetate as the sole source of carbon), completely inhibited protoperithecial differentiation and favoured pure conidial growth (Turian, 1961 a, b). However, in spite of the necessity of a functional TCA cycle and possibly of cytochrome activity for protoperithecial differentiation, formation of protoperithecia may take place under less strict aerobic conditions than are needed for conidial development (Denny, 1933). Suppression of protoperithecial formation may also occur in situations where glyoxylate cycle is not very active. For instance, Westergaard-Mitchell medium supplemented with citrate or succinate induced heavy conidiation in *N. crassa* at 25°C. Under such conditions, the glyoxylate is supposed to be formed due to splitting of some pentoses (Turian, 1963) produced through HMP pathway acting as an alternate oxidative sequence (Turian, 1962 a).

Hirsch (1954) had concluded that nitrate ions seemed to interfere with one or the other stage in the differentiation of protoperithecia and hence exhaustion of nitrate from the medium was a prerequisite to their formation. Amino acids like alanine, glycine and methionine appear to favour conidiation rather than protoperithecial differentiation (Strauss, 1958; Turian and Combettrine, 1963), therefore, it is generally considered that organic nitrogen sources are favourable for conidiation and not for the formation of female sex-organs (Horowitz, 1947; Hirsch, 1954). Addition of ammonium ions, however, leads to the suppression of conidiation (Turian, 1964), which obviously may be due to the known inhibitory action of ammonium ions on nitrate reductase (Kinsky, 1961), the most potent source of NADPH-NADP transformation. This makes reoxidized NADP in short supply limiting the pentose phosphate shunt. Conidiation may also be suppressed by drastically inhibiting carotenogenesis by diphenylamine treatment in *N. crassa* (Turian, 1957), but no definite relationship has been established between conidial formation and biosynthesis of carotenoids (Haxo, 1949). On the other hand, some kind of relationship seems to exist between sexual morphogenesis and nucleic



acid synthesis. Owens *et al.* (1958) recorded that the average RNA:DNA ratio was 8:7 in macroconidia, and it tends to be higher in protoperithecial mycelium than in the conidial mycelium.

Information on the morphogenesis of sexual organs in some other ascomycetes are far more fragmentary. Barnett and Lilly (1947 a) found that in *Ceratostomella fimbriata*, perithecial formation was determined by the thiamine concentration of the medium, and only a high ratio of thiamine to other nutrients permitted perithecial production. Similar roles for thiamine have been reported in *C. pluriannullata* (Robbins and Ma, 1942) and *C. variospora* (Campbell, 1958). Another vitamin, biotin was found to be essential for initiation of sex-organs in *Sordaria fimicola* (Barnett and Lilly, 1947 b). Yet another species *viz.* *S. destruens* requires small quantities of phosphoric esters of glucose and fructose for perithecial production (Hawker, 1948).

Physiology of sexual reproduction in yeasts has been probed considerably, coming only next to *Neurospora*. Almost all the diploid yeast cells, sporulate sexually under appropriate conditions. However, biochemically little is known, about the factors which determine whether the 2N nucleus of a cell will continue to divide mitotically or will suddenly switch-over to meiotic type of division. Also, biochemical distinction between the two processes of nuclear division have not yet been fully realised. That acetate as carbon source, favours sporulation in these proto-ascomycetes is known for long (Stantial, 1935; Adams, 1949; Fowell, 1952; Miller, 1959), but recent studies (Croes, 1967 a) have shown that acetate cannot be considered primary inducer of meiosis, but only as a trigger, allowing full expression of the meiotic proteins. Evidences indicate that it is during the premeiotic mitosis that foundations of sporulation are laid and, therefore, attempts to get a breakthrough into the biochemistry of meiosis should be directed to that phase of the growth cycle. Meanwhile attempts to identify the changes in the biochemistry of cells leading to the induction of meiosis are continuing. One of the significant changes during induction of meiosis is the shift in carbohydrate metabolism from fermentation to intense oxidative respiration. With the gradual utilization of glucose, the TCA cycle enzymes become derepressed and there is a gradual shift in the carbohydrate metabolism of the cell which now becomes highly oxidative and thereby suitable for sporulation (Croes, 1967 b). Snider and Miller (1966) considered that in some cases, sporulation may

even occur with the utilization of ethanol, the end-product of fermentation. Croes (1967 b) has proposed the biochemical changes in carbohydrate and protein metabolism during growth phases as well as during sexual sporulation in *Saccharomyces cerevisiae*, which have

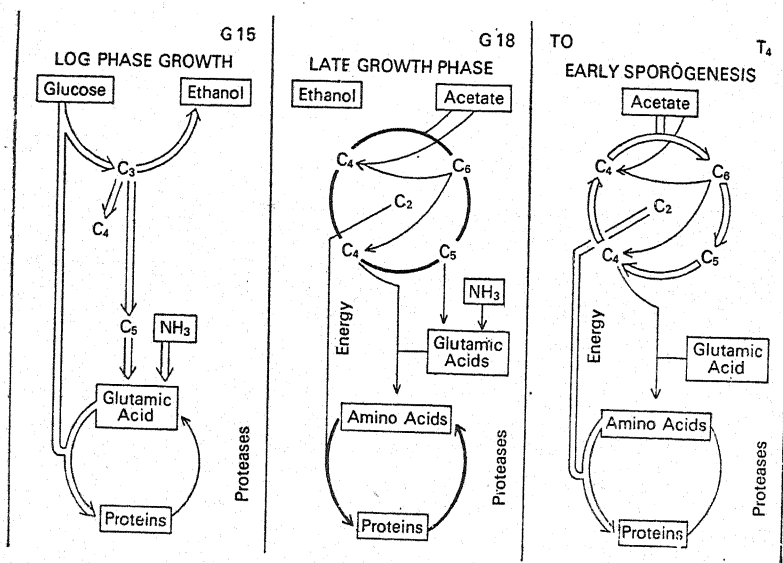


Fig. 14.8. Carbohydrate and Protein metabolism during the two growth phases and early sporogenesis of *Saccharomyces cerevisiae*. The metabolic changes at G 15 and T<sub>0</sub> are considered to induce meiosis. Lines of different thickness mark the relative reaction rates to the process shown (after Croes, 1967 b).

been illustrated in Fig. 14.8. According to him the trigger action of acetate in sporulation probably acts through stimulation of energy-supplying processes in the cell, which results in an insufficiency of the glyoxylate cycle. Further, if a little glyoxylate is added, at the time of transfer of cells to acetate (T<sub>0</sub>), the inhibitory effect of acetate upon mitosis is overcome (Bettleheim and Gay, 1963), and hence the role of glyoxylate in sexual sporulation of yeasts is considered fundamentally (Galbraith and Smith, 1969 b, c) different from its role in asexual sporulation in filamentous fungi.

In basidiomycetous fungi there are no sex-organs, the vegetative hyphae perform the sexual reproductive functions, either by somatic

copulation or by hyphal fusion. Once the plasmogamy is accomplished the secondary mycelium may maintain its dikaryotic nature till the conditions for development of fruit-body or sporophore become favourable. During the development of sporophore karyogamy followed by meiosis takes place in the basidial cell and the haplophase or monokaryon is re-established. Information regarding the factors associated with the initiation and development of sporophore is rather scanty, which may partly be ascribed to the fact that sporophores could not be obtained in cultures for a long time and indeed there was a misconception that these fruit-bodies are produced only under natural conditions. Otherwise, the nutritional requirements of the mycelial phase of growth were already well known (Treschow, 1944).

The limited morphogenetic investigations have covered only the agarics and polypores among the fleshy Basidiomycetes and most of the work has been confined to genera like *Schizophyllum*, *Agaricus*, *Coprinus*, *Collybia* etc. The wood-rotting lignicolous fungus *Schizophyllum commune* has been subjected to extensive nutritional investigations, during the last twenty-five years (Raper and San Antonio, 1954; Raper and Krongelb, 1958; Raper, 1959, 1961 a, b; Leonard and Dick, 1968; Leonard and Raper, 1969). *S. commune* exhibits four developmental phases in its complete life cycle, all of which can occur in a simple medium and under controlled laboratory conditions. Obviously, this has facilitated investigations on this fungus. Typically, production of sporophore of *S. commune* requires establishment of a heterokaryon resulting from fusion of two sexually compatible mycelia (Raper, 1961 a), but recently it has been possible to raise sporophore from certain haploid mycelia, when supplied with exogenous chemical agents obtained from mycelial extracts of *Hormodendrum cladosporoides* and from sporophore extract of *S. commune* and *Agaricus bisporus* (Leonard and Dick, 1968; Leonard and Raper, 1969). A number of other environmental factors have been reported to influence the initiation and development of sporophore e.g., carbon and nitrogen sources (Niederpruem, 1963), light, humidity, aeration etc. Sucrose, maltose, trehalose, cellobiose, glucose, fructose, mannose, galactose, xylose, mannitol, glycerol and ethanol were good sources, while acetate and citrate were unsatisfactory. Among the nitrogen sources, asparagine, glutamine, glutamic acid, serine, alanine, arginine, urea and ammonium phosphate were favourable to sporophore formation. Wessels (1965), on the basis of his studies using a replacement culture technique arrived

at a conclusion that carbon and nitrogen requirements varied at different growth phases of the sporophore-development. External sources of carbon and nitrogen were required at the time of initiation of sporophore primordia, while only carbon source was essential for primordial growth; and during the formation of pilei neither external carbon nor nitrogen was required, suggesting that only endogenous C and N compounds were utilized during the final phase. This indicates that during the final phase there is a flow of materials from mycelium towards the developing fructifications. Light was found to be essential for normal fruiting, while high  $\text{CO}_2$  tension and high humidity inhibited fruiting. Enzymic activities in basidiospores (Niederpruem, 1964; Niederpruem *et al.* 1965; Wessels, 1965; Niederpruem and Dennen, 1966; Aitken and Niederpruem, 1968) as well as homokaryotic and dikaryotic mycelia (Dennen and Niederpruem, 1967) have been compared. It has been observed that NADP-dependent glutamate dehydrogenase remains most active during basidiospore-germination; in homokaryotic mycelium, it shows minimum activity; while in dikaryotic mycelium, both NAD- and NADP- specific glutamate dehydrogenases increase in equal proportions. Conversely, NAD-dependent glutamate dehydrogenase is most active in homokaryotic mycelium. Formation of sporophore-primordia takes place in the presence of exogenous glucose and at that time protein-synthesis takes place at rapid rate. However, RNA synthesis exceeds protein synthesis during the next 24 hours (Wessels, 1965). Pileus formation, on the other hand, starts only after glucose is exhausted. A decrease in the cellular carbohydrate content and a break-down of cell-wall polysaccharide was noted by Wessels (1965), which indicated that endogenous carbohydrate is utilized during this phase. During the development of the pileus, an enzyme R-glucanase acting on insoluble fungal cell-wall R-glucans, initiates the softening or lysis of the cell-wall, which facilitates the expansion of pileus (Wessels, 1969).

Formation of sporophore of *Agaricus bisporus* has been reported to be affected even by the surrounding soil-microflora, (Eger, 1963; Urayama, 1967), and various bacterial species, like *Arthrobacter terregenes*, *Bacillus megatherium* and *Rhizobium meliloti* (Park and Agnihotri, 1969) as well as *Pseudomonas putida* (Hayes *et al.* 1969) have been considered essential. These bacteria produced certain metabolites which could initiate sporophore formation, but the actual initiating factor is yet to be identified. Other environmental factors which affect sporophore development in *A. bisporus* include light,

CO<sub>2</sub> concentration etc. Morphogenetic details of sporophore maturation has been described by Bonner *et al.* (1955).

Environmental as well as nutritional factors affecting sporophore formation have been studied in various other agarics also including *Coprinus* spp., *Collybia velutipes*, *Lentinus* spp., *Armillaria mellea* etc., which have been enumerated by Taber (1966). Recently, Rao and Niederpruem (1969) have compared the activities of certain carbohydrate degrading enzymes in the mono- and dikaryotic mycelia and the sporophore of *Coprinus lagopus*. They could detect the enzymes of hexose monophosphate pathway, sugar alcohol dehydrogenase and trehalase during the entire developmental phase but the proportion of some of them varied during different stages of morphogenesis.

Development of a polypore fructification is strikingly different from the growth-pattern of agarics. *Polyporus brumalis* has been extensively studied by Plunkett (1956, 1958, 1961), who has investigated the role of various nutritional as well as environmental factors influencing sporophore formation. This fungus develops a tubular stipe, which with the help of its growing point grows to some distance before opening out as a hymenial cap. Various physical factors, like pH, temperature, humidity, light etc. have been found to affect fruiting in this fungus. pH and temperature range for sporophore formation in this fungus is unusually narrow (pH 3.1 to 5.1; Temperature 15–25°C), while both dark and light periods are essential for fruiting. Similarly, low humidity and high transpiration rate favoured pileus formation.

Biochemical data on morphogenetic changes in Basidiomycetes in general are scanty. However, the overall information suggests that when some exogenous nutrient becomes limiting, it triggers—the initiation of secondary growth, *i.e.* formation of sporophore. During this phase endogenous nutrients are utilized (possibilities for some exogenous ones can not be ruled out) for the formation of fruiting body. It has been proposed that this phase of secondary growth is regulated by some endogenous growth regulators (Hagimoto and Konishi, 1959; Gruen, 1963), and a recent report of growth promoting effect of some growth substances, like indoleacetic acid and gibberellin on agarics (Volz and Beneke, 1969) seems to substantiate this possibility.

SPORE GERMINATION

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Fungi are characterized by a wide variety of spores, possessing a complex morphology and specialized physiology, which make these microscopic bodies much more enduring than the vegetative thallus. Moreover, produced at the climax of the fungal life-cycle, spores have to perform embryonic functions for which they are equipped with the inherent potentiality to develop into a new generation. Spores being the principal agent of fungal dispersal have received consistent attention of research-workers. The topic of spore-germination in fungi has been discussed right from the time of DeBary (1887), in a number of books and review articles, including those by Gottlieb (1950), Lilly and Barnett (1951), Cochrane (1958, 1960), Shaw (1964), Allen (1965), Sussman (1965 a, 1966 a, b), as well as Sussman and Halvorson (1966).

Events directly involving fungal spores are multiple, including their (i) formation, (ii) discharge or release, (iii) dispersal to new localities, (iv) period of dormancy, if any, and (v) germination into a new thallus. Here, only the final event, *i.e.* germination will be discussed. Germination of fungal spores is essentially a process during which the normal metabolic and physiological activity is restored after a temporary halt or check in these activities in the resting spore, followed by a morphological transformation of the spore into a new thallus. In a general sense, however, germination usually implies the emergence of a definite germ tube. In some cases, germination does not produce any germ tube, rather it leads to the cleavage of the protoplasm into a number of cells, which develop into zoospores as they emerge from the zoosporangium. However, the formation of a typical germ tube occurs in these cases also, when the zoospores undergo germination after they become nonmotile. Other morphological variations may also be noted in specific groups of fungi, but these are of little relevance in the context of the present discussion,

which will mainly be concerned with physiology and biochemistry of germination. Of course, a brief treatment of dormancy and its physiological causes will also be incorporated.

## DORMANCY

Dispersal of spores may lodge them in an environment which may or may not be conducive to their germination and the growth of the resultant thallus. It has been observed that some of the fungal spores initially remain indifferent to the environment around them, and even if the conditions are favourable for their germination, they do not do so for a specified period of time. Such spores are said to be dormant, and during the period of dormancy, they are supposed to complete their maturation process, or if already mature this period is treated as an enforced period of rest. This kind of dormancy is obviously controlled by certain internal factors of the spore and is, therefore, designated as constitutional dormancy. This is in contrast to the exogenous or the environmental dormancy, which is controlled and enforced upon the spore by some external environmental factors, unfavourable for germination and the growth of the new thallus. Exogenous dormancy is obviously meant to tide-over the hostile environment in which the spore has been lodged, and it is broken as soon as the conditions for germination improve.

### Constitutive Dormancy

This type of dormancy is exhibited by the ascospores of certain Ascomycetes, and has been studied in *Neurospora*. Doran (1922) considered that the internal factors controlling constitutive dormancy included the maturity of spores, its longevity, "animation" as well as vitality. Shear and Dodge (1927) as well as Goddard (1935) observed that the dormant ascospores of *Neurospora* could be induced to germinate by giving them a heat treatment for 20 minutes at 60°C. Sussman (1965 a) has enlisted barrier to the penetration of nutrients, a metabolic block and the production of self-inhibitors as the possible innate properties of a dormant fungal spore. Discussion on the dormancy-factors will however be restricted here on (i) permeability, (ii) self-inhibitors and (iii) metabolic block:

(i) *Permeability*. The water-content of a dormant spore is generally too low to allow active metabolic processes. Therefore, entry of water either in the liquid state or gaseous phase seems to be essential for



initiation of germination, which is long known to be marked by enhanced metabolic activities. There are of course exceptions, *e.g.* water is inimical for the germination of conidia of powdery mildews, while those of downy mildews are able to germinate only in the presence of liquid water. Yet, it has been a common concept that non-permeability of the spore-wall to water and the dissolved substances is the principal cause of dormancy, and attempts to initiate germination by causing suitable changes in the spore wall has been made by several workers. Even, efforts to correlate the extent of the dormancy-period with the thickness of spore-wall were made. It was often observed that spores which were allowed to form abnormally thin spore-wall under suitably controlled conditions, exhibited a marked reduction in their dormancy-period (Brierly, 1917; Gwynne-Vaughan and Williamson, 1933; Stiiben, 1939). Further evidence was drawn from the observations that prior to germination, the spore-wall undergoes autodigestion (Blackwell, 1935; McKay, 1939), or it cracks (Cantino, 1951; Machlis and Ossia, 1953). Treatments of the spore-wall with appropriate enzymes appeared to enhance germinability (Brierley, 1917). In some recent studies by Salvatore *et al.* (1973) and Stanghellini and Russell (1973), enzymes from snails (*Helix aspersa* and *Planorbis* sp.) have been used, and they were found to enhance abruptly the germination percentage of oospores of *Phytophthora* and *Pythium* respectively.

Dormant ascospores of *Neurospora* were found to be freely permeable to water (Sussman, 1954) but impermeable to cations, and also to anions with more than two carbon atoms (Sussman, 1966 b). Conidia of the same fungus were, however, freely permeable to both inorganic and organic substances, and they do not show constitutional dormancy. When the dormant spores of *Phycomyces blakesleeianus* were activated to germinate by acetate treatment, a drastic change in the permeability of spore-wall to heavy metals was noted (Borchert, 1962).

Later investigations have shown that the mechanism of water uptake by spores is rather complex, and is not simply governed by the permeability factor. Permeability may be a primary requirement, but the in-flow of water seems to be a non-osmotic and energy-requiring process. In a few fungi, like *Fusarium*, *Aspergillus* (Yanagita, 1957; Marchant and White, 1966); and some Mucoraceae (Wood-Baker, 1955) the initial phase of water-absorption has been claimed to be non-metabolic, but generally glucose has been found to be necessary for swelling in these fungi, which suggests that metabolic activity is

essential for water-uptake. Further, the osmotic pressure of the glucose solution had no effect on water absorption which indicates its non-osmotic mechanism. Marchant and White (1966) have found that the macroconidia of *Fusarium* could attain maximum swelling only when glucose and a nitrogen source were present, which also indicates that an active absorption of water is involved.

(ii) *Self-inhibition*. Edgerton (1910) noted that when more than 12 to 15 conidia of *Colletotrichum lindemuthianum* were placed in 1 cubic millimeter of the medium, the germination percentage declined. Boyd (1952) found that in *Fusarium caeruleum*, conidial germination was as low as 1%, when 2,000 conidia per field were included. Failure of the fungal spores to germinate when present in large number has been attributed to the presence of some kind of self-inhibitors in the spores. It is suggested that these inhibiting substances might originate in the parent plant during sporogenesis, and are carried by the spores. Washing the spores in water is supposed to get rid of the inhibiting substances, because it allows germination, and water extracts of spores are highly inhibitory to germination. Self-inhibitors have been thought to be present in the spores of various parasitic fungi, including *Erysiphe graminis* (Domsch 1954), *Peronospora manshurica* (Dunleavy and Snyder, 1963) *Puccinia* spp. (Allen, 1955; LeRoux and Dickson, 1957), *Uromyces phaseoli* (Yarwood, 1954; 1956 a), etc. Among the saprophytic fungi self-inhibitor mechanism has been indicated in conidia of *Glomerella cingulata* (Richardson and Thorn, 1962) and species of *Neurospora*, *Aspergillus*, *Penicillium*, *Fusarium*, *Phycomyces* and *Sclerotinia* and in sporangiospores of *Rhizopus* and *Mucor* (Cf. Cochrane 1958; Allen, 1965) as well as *Geotrichum candidum* (Steele, 1973). The concept of self-inhibitors inhibiting spore-germination and causing dormancy has led to the development of a theory that the spores must also contain some self-activators which may counteract the effect of inhibitors to allow germination. This concept is based on the counteracting action of some chemicals, like aldehydes and alcohols. Besides, steroids and surface-active compounds like saponins are also effective germination-stimulants (Weintraub *et al.* 1958). Interestingly, furfural and some other heterocyclic substances, some of which are metabolic poison, also stimulate germination. Moreover such stimulating compounds like coumarins, phenols, etc. have been isolated from fungal spores (Allen, 1957; French *et al.* 1957; French and Weintraub, 1957; Van Sumere *et al.*

1957). Sussman and Holvorson (1966) have suggested that the activators may well operate by a direct competition with the inhibitors or they may interfere either with the synthesis or release of the inhibitors. Under natural conditions it is the balance between inhibitors and stimulants, which probably regulate spore-germination.

The chemical nature of the inhibiting substance is unknown for most of the spores. Allen (1955) made a pioneering effort to study the properties of self-inhibitors of uredospores of *Puccinia graminis* f. sp. *tritici*, from which some non-volatile inhibiting substances were later isolated by Van Sumere *et al.* (1957). These included caffeic acid, p-hydroxybenzoic acid, ferulic acid and vanillic acid, but only ferulic acid inhibited spore germination at all the concentrations (upto 1 ug/ml). Allen's (1955) observation that the inhibiting influence was effective even across an air-gap suggested that the uredospores of *P. graminis* f. sp. *tritici* also produced some volatile inhibitor. Forsyth (1955) also came to a similar conclusion, and found that the inhibiting activity of the uredospores was identical to that of trimethylethylene. Moreover, some other observations, like similar means of counteracting their inhibitory influence, identical absorption spectra in acetone, etc. indicated that the uredospore-inhibitor might be trimethylethylene itself. This possibility is further supported by the isolation of trimethylamine from the spores of *Tilletia caries*, which inhibits spore germination in low concentration, and hence it is considered as the endogenous inhibitor of smut spores (Ettel and Halbsguth, 1963).

Self-inhibitors have been thought to be formed in sporangium itself, because spores generally do not germinate within them (Von Stosch, 1935). Some isolated instances of spore-germination inside the sporangia (Chamberlain and Allison, 1945; Schnathorst, 1959) appear to be more an exception than a rule. This way, self-inhibition confers upon the spores a distinct ecological advantage, because this prevents them to germinate precociously and thereby helps them in their efficient dispersal.

(iii) *Metabolic control.* Dormancy of fungal spores has also been attributed to some kind of a metabolic block or to some nutritional deficiency. It has been a common observation that dormant spores may be made to germinate if they are activated by certain treatment(s) or if some nutrients are provided, which are ordinarily not required for vegetative growth. Most of such activators serve as a "trigger" which

affects such a change in the metabolism of the dormant spore, that it initiates germination. It was Goddard and Smith (1936), who for the first time attempted to identify the metabolic changes caused by such "triggers". They noted that increase in the anaerobic production of  $\text{CO}_2$  by *Neurospora tetrasperma* ascospores, was proportional to the number of germinating spores. From further experiments they concluded that the dormant ascospores lacked the enzyme pyruvic carboxylase, which imposed a metabolic block. The activated spores, on the other hand, contained this enzyme which permitted their germination. However, Sussman *et al.* (1956) observed that pyruvic carboxylase activity was present in dormant, activated and germinating ascospores, although the activity was lower in the former than in the latter two cases. They also recorded almost equal amounts of the coenzyme diphosphothiamine in dormant as well as germinating ascospores. Sussman and his associates (Lingappa and Sussman, 1959; Sussman, and Lingappa, 1959; Hill and Sussman, 1964; Sussman, 1966 a, b) have proposed an alternative mechanism of control of dormancy of *Neurospora* ascospores. According to their hypothesis, activation of ascospores leading to germination may cause any of the following changes: (i) an inhibitor of a trehalose-degrading enzyme is destroyed, (ii) the trehalose degrading enzyme is synthesized from its precursor, the conversion being analogous to the trypsinogen-trypsin transformation (iii) the enzyme is thought to be spatially separated from its substrate inside a dormant spore, and activation may bring the two together, (iv) a series of interlocking enzymic reactions are shifted from one steady-state level to another as suggested by Delbruck (1949).

Requirement of some specific nutrient for spore-germination was first noted by Schopfer (1942) in *Rhizopus suinus*, where the zygospore germination required *meso*-inositol. This substance is not required for the vegetative growth of *R. suinus*. Some other growth factors, like niacinamide (Von Guttenberg and Strutz, 1952), thiamine (Hawker, 1950), as well as L-prolin and L-alanine (Yanagita, 1955) have also been reported to stimulate germination. J.C. Cochrane *et al.* (1963) have studied the germination requirement of spores of *Fusarium solani* f. *sp. phaseoli*. Spores of this fungus germinate only when exogenous sources of carbon and nitrogen as well as yeast-extract are provided, the latter may, however, be replaced either by ethyl alcohol or acetone. Role of ethyl alcohol in germination has been suggested to furnish active acetaldehyde, which is essential for synthesis of certain amino acids required for germination of spores.

Haware and Pavgi (1972), however, reported that external nutritional factors did not affect chlamydospore germination in *Protomyces* species, although various acids, alkalies,  $\text{KMnO}_4$  and fertilizers induced and stimulated their germination. Similarly, oospore germination in *Pythium hydroporum* could occur in distilled water (AlHassan and Fergus, 1973). But it is yet to be ascertained, whether these nutritive substances are required specifically for spore-germination only or for mycelial growth as well. If the latter is found to be the case, attempts to find out some other explanation of the metabolic block causing dormancy should continue. Allen (1965) suggests that the qualitative as well as quantitative requirements of nutrients for spore-germination are determined by the cultural history of sporulation.

It is obvious, therefore, that such nutritional data may not provide any definite evidence in support of a particular mechanism operating in a metabolically controlled dormant spore, and hence studies at specific enzymic level are essential. Sussman (1966 a) has indicated the inherent difficulties underlying such studies but at the same time has advocated for such investigations during different phases, both prior to and after the germination of spore.

### Exogenous Dormancy

Various environmental factors may enforce dormancy on a spore, which is otherwise ready to germinate. Physical factors, like temperature, humidity, pH,  $\text{O}_2/\text{CO}_2$  balance (in soil), etc. may have definite influence over the germinability of fungal spores some of which have been discussed in chapter XIV. Some of the chemical factors influencing germination are considered here.

(i) *Chemical inhibitors.* Different chemical compounds of varying origin may be present in different environment, which exert inhibitory influence upon germination of fungal spores and impose upon them exogenous dormancy. In soil, fungal spores are maintained in a dormant condition due to widespread occurrence of soil-fungistasis. This phenomenon is present in almost all types of soils, except in some deep sub-soils, highly acid soils or soils exposed to cold temperatures. Although, it is generally agreed upon that some mycostatic substances exist in soil, yet there is no clearcut understanding of the mechanism involved, for which divergent views have been expressed from time to time. Lockwood (1964) suggested that the living micro-organisms compete for nutrients required for the germination of fungal spores, which are thus unable to germinate

unless the nutrients are made available from a newly incorporated substrate. According to this view, therefore no inhibitor as such is involved. However, specific inhibitors produced by soil micro-organisms has been suggested to cause fungistasis by Waid (1960) and Park (1967). Yet another explanation of fungistasis envisages the involvement of certain staling substances produced by soil microflora (Park, 1960; Jackson, 1965).

In fact, fungistasis appears to be a complex phenomenon caused by a multitude of factors, but ultimately owing to the soil microbial population. Evidences indicate that possibly nutrients, as well as inhibitory substances (like antibiotic, staling products etc.) both may be responsible for fungistasis in the same or different soils. Observations like reversal of fungistasis due to addition of readily decomposable organic material like glucose, or due to growth of plant-roots which are known to provide nutrients in the form of root-exudates and detritus, go to suggest that some nutritional factors are involved in fungistasis. On the other hand, reversal of fungistasis due to sterilization of soil and its subsequent restoration due to addition of a pinch of unsterilized soil, as well as the general lack of fungistasis in soils which are without a substantial microbial population indicate that it is due to some microbial activity, probably due to some inhibitory metabolites produced by them. However, uptill now no inhibitory substance has been unambiguously demonstrated to exist in the soil. The suggestions of Lingappa and Lockwood (1961) that the fungal spores provide nutrients to the soil-microbes in their immediate vicinity, which grow and produce antibiotics to cause fungistasis, is also without any supporting data, and it seems that the question is still wide open for investigation.

Fungistatic principles may also be present in other habitats, like marine environment (Borut and Johnson, 1962) but practically nothing is known of their chemical identity.

Exogenous dormancy may also be caused by the host plants, which inhibit the germination of spores of pathogenic fungi as a consequence of host-resistance. A variety of plant-products, including phenols, mustard oils etc. have inhibitory effects upon spore-germination. It is now well documented that surfaces of plant leaves contain some chemical substances, which inhibit the germination of spores of pathogenic fungi (Kovacs and Sieoke, 1956; Wain, 1957). However, the most conclusive evidence of a chemical inhibitor pro-

duced by the host tissue acting against the germination of a pathogenic fungus (*Colletotrichum circinans*) has been obtained in case of onion. The work, which has led to the identification of the inhibitory substance as catechol and protocatechuic acid has been reviewed by two of the principal investigators (Walker and Stahmann, 1955).

Similarly, evidences though not conclusive, have been adduced (Buxton 1957 a, 1962) to suggest that root-exudates of resistant varieties of some plants exert inhibitory effects upon germination of conidia.

### PHYSIOLOGY OF GERMINATION

Germination of spore involves a series of irreversible morphological, cytological and physiological changes, which ultimately lead to the transformation of the relatively inactive spore into a highly active vegetative thallus. We will restrict here our concern to the physiological and biochemical changes, in the spore in course of germination. Morphological and cytological aspects have been dealt in detail by Hawker (1966), Sussman (1966 a) and Fuller (1966).

#### Metabolic Changes in Germinating Spore

The ultimate object of germination is obviously the formation of new protoplasm for the ensuing vegetative growth. Although, spores are inherently equipped for most of the synthetic processes required for formation of new protoplasm, yet they are characterized by their low metabolic activity. Even the respiration rate is quite low in resting spores. Therefore, it may easily be conceived that the initiation of germination stimulates the metabolic activity of spores, and the whole biosynthetic machinery is so geared up as to meet the demand of a germinating spore, developing fast into a growing mycelium. It is also obvious that a spore, although low in activity, is possibly packed with the requisites of a highly active metabolism and complex biosynthetic reactions. Most ideal condition would be the presence of:

- (i) a readily available energy and carbon source,
- (ii) a complete set of enzymes,
- (iii) various TCA cycle intermediates,
- (iv) a pool of amino acids for protein synthesis,
- (v) nucleic acids or their components, etc.

However, such an ideal biochemical store is seldom provided in a



fungal spore and, therefore, the germination of spore is often temporarily blocked either for want of a specific nutrient or due to lack of an enzyme or for want of a triggering action of some environmental factor. Moreover, spores contain very little of water, which allows the metabolic activities to proceed only at a minimal rate. Therefore, in most of the spores, absorption of water is the first prerequisite for germination, which is followed by increased rate of respiration, if other environmental conditions, particularly the temperature remains favourable.

(i) *Respiration.* Striking increase in the respiratory activity of germinating spores was first noted by Goddard (1935) and Goddard and Smith (1938) in *Neurospora tetrasperma* ascospores. Mandels *et al.* (1956) demonstrated that during germination, spores of *Myrothecium verrucaria* exhibited higher rate of respiration, which increased linearly with time. Similar results have been obtained with several other fungi also, including *Aspergillus oryzae* (Terui and Mochizuki, 1955), *A. niger* (Yanagita, 1957) and *Phycomyces blakesleeana* (Halbgsuth and Rudolph 1959; Rudolph 1960). Even the uredospores of *Puccinia graminis* f. sp. *tritici*, which were previously considered an exception to this generalisation, have now been shown by Allen (1963) to exhibit similar response under certain conditions. V.W. Cochrane *et al.* (1963 b, c) found that germinating macroconidia of *Fusarium solani* f. *phaseoli* exhibited enhanced respiration only when exogenous glucose was provided and not with ethanol or mannose. Such results possibly indicate that increase in the respiration rate occurs only when other conditions are conducive to spore germination. It has been observed that mere wetting of the spores does not generally result in enhanced respiratory rate, particularly in case of those spores, which need certain specific exogenous nutrient for germination. For instance, spores of *Aspergillus niger* (Yanagita, 1957), *Fusarium roseum* (V.W. Cochrane *et al.* 1963 b), *Rhizopus arrhizus* (Weber and Ogawa, 1965), etc. do not show any increase in their respiratory activity, when simply placed in water, owing to their requirement for nutrients to germinate. On the other hand, spores of obligate parasites, when placed in water or under humid conditions, show enhanced respiration, obviously because they are able to germinate under such conditions at the expense of their endogenous food-reserve.

Germination of most fungal spores is strictly an aerobic phenomenon and hence for normal germination availability of oxygen is essential.

Germination is retarded or even stopped if deprived of oxygen (Wood-Baker, 1955; J.C. Cochrane *et al.* 1963). However, changes in oxygen-availability probably cannot initiate germination.

Attempts have also been made to study the effect of germination stimulant as well as germination-inhibitors on respiration. Available reports indicate that stimulants enhance respiration and it seems possible that these compounds actually stimulate germination-inhibitors, however it is not yet clear whether these inhibitors act directly on respiratory metabolism or somewhere etc. This aspect needs further attention.

(ii) *Energy Requirements.* Energy is one of the primary requirements of spore-germination, and is furnished by the oxidation of either the endogenous food-reserves or the exogenously supplied carbon-sources, such as glucose, which may easily be oxidized by the spore. However, it is now believed that the energy requirement for synthetic reactions occurring in the spores at an early stage of germination, can hardly be fulfilled by the catabolic degradation of glucose. This is because, at such an early stage of germination, the various catabolic sequences, like EM and HMP pathways as well as the terminal electron transport system are themselves at a low ebb, and they increase only when the germination progresses. Consequently, the ATP required for the synthesis of nucleic acid and enzyme-proteins during the early phases of germination is in short supply. To meet their high energy requirement right from the onset of germination, the fungal spores are equipped with alternative sources. These include polymetaphosphates, and phospholipids, which have been shown to be present in the spores as a ready source of stored energy in a number of fungi. These compounds release their stored energy by a simple hydrolysis and provide for the synthesis of ATP molecules. Evidences to support such possibilities have been obtained experimentally with radioisotopic  $p^{32}$ . Nishi (1961) noted in the conidia of *Aspergillus niger* that the quantity of labelled polyphosphates and phospholipids declined with the progress of germination, while there were simultaneous increases, primarily in the labelled nucleotides and sugar phosphates including AMP, ADP, ATP and GMP (guanosine monophosphates) as well as glycerophosphate, and later in the labelled RNA.

In the later stages of germination, however, the energy furnished by the catabolic degradation of carbon compounds is generally sufficient to meet the requirements.

(iii) *Metabolic activities.* The onset of germination leads to enhanced metabolic activities, for which suitable substrates are needed. The spores of some fungi utilize their endogenous food-reserve to sustain these activities, while those needing external supply of nutrients utilize exogenous compounds. Utilization of the two types of substrates are described separately:

(a) *Endogenous substrate.* Spores utilizing endogenous reserves for germination, usually contain at maturity all the essential requisites for growth upto a short germ tube or even upto the development of the new thallus to a certain extent. Ascospores of *Neurospora* and *Ascobolus*, teliospores of rusts, Smut-Spores and resting sporangia of Phycomycetes are the examples of such types.

Analysis of spore-content of several such fungi has indicated that a large variety of substrate-types is present in the spore. Even different spore types from the same fungus, and same spore-type from different strains, species or genera of fungi differ in their food-content. Recently, Tulloch and his associates have made interesting observations regarding the lipid content of stem rust uredospores and that of other spores of the rust-fungi (Tulloch *et al.* 1959; Tulloch, 1960, 1963, 1964; Tulloch and Ledingham, 1960, 1962, 1964). They noted that although uredospores, teliospores and basidiospores produced by a particular rust species had a very similar composition of spore-oil, yet the aeciospores were sometimes markedly different. They also could not find any appreciable difference in the composition of spore-oil of different races of wheat-stem rust, although they are known to differ significantly in their amino acid content (McKillican, 1960). It may be noted that lipids seem to play a very important role in the germination of rust uredospores, because they possibly serve as the substrate for their germination, and are depleted rapidly during germination (Shu *et al.* 1954). They demonstrated that in germinating uredospores of *Puccinia graminis* f. sp. *tritici* endogenous fats and proteins were utilized. Similarly, uredospores of *Melampsora lini* also metabolize fat (Frear, 1960).

Other organic compounds detected in fungal spores include organic acids, amino acids, soluble sugars, polyalcohols etc. Malca *et al.* (1962) found malate as the principal organic acid and glutamate, asparagine and glutamine as the prominent amino compounds besides some free reducing sugars in the conidia of barley powdery mildews. Later, Edwards and Allen (cited by Allen 1965) detected arabitol and mannitol in these conidia. These polyalcohols along-

with glycerol were also found to make about 12% of the dry-weight of uredospores of *Puccinia graminis* (Reisener *et al.* 1962). D-Mannitol is also present in the spores of *P. coronata* (Tani and Naito, 1960). However, information on the metabolic changes occurring in rust uredospores are limited, and the available reports (Tani and Naito, 1960; Reisener *et al.* 1962; Caltrider *et al.* 1963) indicate that during incubation the uredospores primarily utilize fatty acids and polyalcohols, but during the growth of the germ tube the lipid content decreases and the carbohydrate content increases. Information on metabolic changes in powdery mildew conidia is, however, lacking.

Studies on various other fungal spores including those of *Fusarium solani*, (V.W. Cochrane *et al.* 1963 b), *Glomerella cingulata* (Jack 1964), *Penicillium atrovirens* (Van Etten, 1964) and *Neurospora sitophila* (Owens *et al.* 1958) have shown their high lipid content, which decreases during incubation, showing their possible role during germination. Lingappa and Sussman (1959), however, reported that the dormant ascospores of *Neurospora* spp. use lipids, and on being activated to germinate, they shift to utilization of soluble carbohydrate, which was later identified as trehalose (Sussman and Lingappa, 1959; Sussman, 1961 a). Nevertheless, lipid utilization continues together with that of trehalose, and after the exhaustion of the latter, the lipid break-down is the only source of energy. Sussman (1966 a) has, therefore, concluded that many of the fungal spores might be utilizing both lipids and carbohydrates during germination.

(b) *Exogenous substrate.* Some fungal spores require exogenous nutrients for germination, although they do contain endogenous food-reserve. Conidia of *Fusarium solani* (J.C. Cochrane *et al.* 1963) and *Myrothecium verrucaria* (Mandels, 1963) contain sufficient endogenous food-reserve, yet they require specific exogenous nutrients to germinate. Oku (1960) reported that starved conidia of *Cochliobolus miyabeanus* germinated at the expense of a variety of exogenous sugars as well as an endogenous glucan.

It has also been noted that the mode of utilization of exogenous carbon-source may vary at different stages of spore-germination. Such a change has been observed in the pathway of glucose metabolism in macroconidia of *Fusarium solani* (V.W. Cochrane *et al.*, 1963 a), teliospores of *Tilletia caries* (Newburgh and Cheldelin, 1958) and *Ustilago maydis* (Caltrider and Gottlieb, 1963). It was noted that in *Fusarium solani* and *Tilletia caries*, spores prior to germination produced more CO<sub>2</sub> from carbon-1, as compared to germinating spores or the mycelium. Further, with the advance-

ment of germination, spores of all the three fungi could produce more and more of anaerobic  $\text{CO}_2$ . In contrast, resting spores of none of these fungi produced any  $\text{CO}_2$  anaerobically. Similar differences in the metabolic activities of dormant and germinating spores have been noted in case of steroid-oxidation. Spores of many such fungi, which affect oxidation of steroids, are capable to carry out only a single reaction prior to germination, but after germination, further degradation of steroids into a variety of end-products takes place. Similarly, in *Penicillium roquefortii* (Gehrig and Knight, 1961), only the conidia have the capacity to carry out a decarboxylation of fatty acid to produce a 2-Ketone, but as they germinate, they lose this ability (Gehrig and Knight, 1963).

Metabolic activities during various stages of germination have also been compared and the available reports indicate that in the very beginning of the germinating process, the catabolic reactions are more active than the synthetic ones. During this phase, the carbon-reserves of the spore are oxidatively catabolised primarily for the release of energy. At early stage the spore is not able to utilize the exogenous glucose in amounts sufficient to sustain synthetic reactions to any considerable extent, and, therefore, there is a small decline in the dry-weight of the spore. Ohmori and Gottlieb (1965) recorded slight decrease in the dry-weight of spores in three of the four fungi they studied. The decline noted during the first two hours of incubation was followed by a continuous increase in their dry-weight till the time of maximal percentage of germination when the spores weighed four times their original weight. Increase in dry-weight has also been reported earlier by Mandels and Norton (1948), and Mandels (1953), in spores of *Myrothecium verrucaria* incubated in nutrient solution. All such reports including those of Horikoshi *et al.* (1965) for *Aspergillus oryzae* and that of V.W. Cochrane *et al.* (1963 b) for *Fusarium solani* suggest that after an initial predominance of catabolic reactions, the spore-metabolism shifts to the synthetic phase. During this phase the spore utilizes the exogenous nutrient in sufficient quantity to support the biosynthetic reactions then predominating in the germinating spore.

Predominance of synthetic reactions during the later phase of germination is also indicated by the observations (Niederpruem, 1964; Ohmori and Gottlieb, 1965) that consumption of oxygen per unit substrate absorbed during this phase remains too low to affect complete oxidation of the substrate. In such a situation either the

incompletely oxidized intermediates will accumulate or the carbon moieties will be utilized for biosynthetic reactions.

### Enzyme Complement

Before describing the different biosynthetic processes occurring in a germinating spore, it is desirable to assess its enzymatic capabilities as well as the possibilities of induction of new enzymes, when needed.

For germination, spores usually need almost the same complement of enzymes which is needed for normal cell respiration and growth. In fact, respiratory enzymes of major pathways of respiration have been shown to be present in spores of a number of fungi, although in minimal quantities, but their amount increases with the progress of germination. Enzymic studies on some obligate parasites have also indicated that their spores contain most, if not all, of the common enzymes found in other fungi and higher plants (Shu and Ledingham, 1956; Gottlieb and Ramchandran, 1960; Caltrider and Gottlieb, 1963). However, there are certain reports which suggest that some important respiratory enzymes lack both in ungerminated spores as well as in spores at an early stage of germination. Bhatnagar and Krishnan (1969 c) found conidia of *A. niger* devoid of phosphoglucomutase, phosphohexokinase and aldolase, all belonging to EM pathway. These enzymes could be synthesized only after twelve hours of incubation of the conidia, when they had already started to form germ-tubes. *Ustilago maydis* spores were at a greater disadvantage as they lacked as many as seven different enzymes belonging to EM and HMP pathways as well as the TCA cycle (Gottlieb and Caltrider, 1963). Synthesis of these enzymes too did not take place before completing ten to twelve hours of incubation.

Although studies dealing with the changes in the enzymic activities during germination of spores have received limited attention, the available reports, however, indicate that new enzymes do appear during germination, while a few disappear and others remain unaltered. The changes in metabolic activities of some spores during germination, however, do not necessarily reflect the synthesis of new enzymes, rather it may be due to a change in the activity of those already present in the spore.

Increase in activity during germination has been shown for many enzymes. Activity of sucrase in spores of *Myrothecium verrucaria* increased with the progress of germination (Mandels and Norton, 1949). Trehalase content in the ascospores of *Neurospora crassa* increased by 400% within two hours of activation by heat (Hill and

Sussman, 1964). However, no increase in the activity of mannitol dehydrogenase was recorded during conidial germination of *Aspergillus oryzae* (Horikoshi *et al.* 1965), and in fact Bhatnagar and Krishnan (1960 a) recorded decline in the activity of catalase during germination of *A. niger* spores. Despite this variation in the synthesis and activity of some specific enzymes, the available information indicate that during germination the overall enzyme-protein synthesis takes place at a higher rate than the normal protein.

### Biosynthetic Processes during Germination

Experiments with labelled substrates have furnished ample evidences to suggest that varied synthetic reactions take place in a germinating spore. Incubation of spores in a medium containing labelled acetate produced labelling in a variety of spore contents, including organic acids, amino acids, sugars, proteins and nucleic acids. However, the pattern of labelling varied in different fungal spores, e.g. spores of saprophytic fungi exhibited much higher labelling of proteins than those of obligate parasites (Staples *et al.* 1961 a). Similarly in uredospores of *Uromyces phaseoli* the organic acids acquired labelling very quickly. Such variations in the synthetic abilities of different fungal spores may be attributed to their enzyme-complement.

*Synthesis of carbohydrates.* When labelled 2-C<sup>14</sup>-acetate was supplied as an exogenous carbon-source, labelled sugars could be detected in the germinating spores of rusts and saprophytic as well as facultatively parasitic fungi. Several free sugars including trehalose, ribose, fructose, mannose, sorbose and sugar alcohols like mannitol were formed (Staples *et al.* 1962). Of these, trehalose was the principal sugar in the spores of *Uromyces phaseoli* and *Puccinia sorghi*. However, in *P. graminis tritici* glucose was the primary sugar synthesized by the germinating uredospores (Caltrider and Gottlieb, 1963), while in spores of *Aspergillus niger* and *Glomerella cingulata* sucrose was dominant. Polysaccharides with ribose, xylose, galactose and maltose units have also been detected in germinating spores (Townsend and Bell, 1965).

All these sugars are possibly synthesized from acetate through the glyoxylate cycle. The malate produced due to condensation of glyoxylate and the 2-carbon acetyl fragment is probably decarboxylated to form phosphoenolpyruvate, which enters the EM pathway and may ultimately lead to the synthesis of glucose. The HMP pathway may also be playing its part in the production of pentoses,



hexoses as well as of sugars with 3, 4 and 7 carbon atoms. The concerned enzymes have been demonstrated in various fungal spores.

*Lipid synthesis.* It was mentioned earlier, that lipids constitute the major endogenous substrate, which are utilized by the spores to sustain the early processes of germination. It is, therefore not surprising that during germination, lipid content of spore either do not increase to a considerable extent, or even decrease. In spores of obligate parasites, like *Puccinia graminis tritici* and *Uromyces phaseoli*, endogenous lipid reserves are utilized to derive energy and, therefore, their level goes down. Caltrider *et al.* (1963) noted that the uredospores of these rust fungi lost fatty acids with the advancement of germination.

Some spores needing exogenous substrate to germinate also show similar response. In *Aspergillus nidulans* lipid content continued to decline in germinating conidia, till it was undetectable (Shepard, 1957). Similar observation was recorded by V.W. Cochrane *et al.* (1963 b) in *Fusarium solani* f. *phaseoli*.

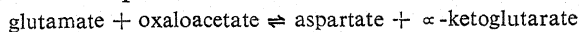
In contrast, there are certain reports to suggest that during germination lipid-synthesis may also occur (although to a limited extent only), atleast in some fungi, e.g. *Fusarium roseum* (Sister and Cox, 1954), *Penicillium atrovenetum* (V.W. Cochrane *et al.* 1963 b), *Curvularia geniculata* (Townsend and Bell, 1965), etc.

It must be noted here that in most of the work quoted above it is only the fatty acids and fats, whose quantitative decline has been reported and not the entire lipid content. In fact, lipid content as a whole was found to double within a 24 hours germination period in *Penicillium atrovenetum* (Van Etten and Gottlieb, 1964). Caltrider *et al.* (1963) have reported that only the fatty acid content of the rust-spores declined during germination, while another-lipid fraction which they called the "non-saponifiable" fraction, was actually synthesized during germination.

Synthesis of lipids in germinating fungal spores takes place by routes known for fungal mycelia. Isotopic experiments with spores of *Aspergillus niger* indicated that the amino acid alanine is deaminated to form pyruvate, which by the known steps of its oxidative decarboxylation may provide the acetyl fragments needed for fatty acid synthesis.

*Synthesis of amino acids.* That new amino acids are synthesized to meet the demands of protein synthesis during germination is well established. The amine groups required for the synthesis of new amino acids are furnished by the internal pool of amino acids present in the

spore. The endogenous pool of a fungal spore is created at the time of sporulation itself. In most fungal spores, glutamic and aspartic acids or their respective amides are the major storage amino-compounds. The presence of different transaminases in spores suggests that the new amino acids are synthesized chiefly by transamination of the endogenous amino compounds. In the cell-free extracts obtained from uredospores of *Puccinia helianthi*, the following transamination reaction could take place in reversible manner.



In *Aspergillus nidulans*, Shepard (1957) recorded rapid fall in the concentration of glutamine, asparagine and aminobutyric acid during first hour of germination alongwith simultaneous rise in the quantity of glutamic and aspartic acids. This indicates the intermediate role of amides in transamination. However, in *A. niger* transaminases were synthesized only after 3 hours of incubation of spores (Takabe and Yanagita, 1959) and possibly on this account spores of this fungus require exogenous L-alanine or L-proline as nitrogen source for germination. Although definite evidence is lacking, yet a similar proline requirement in the spores of *Rhizopus arrhizus* has been attributed to a proline deficiency and the lack of appropriate enzymes for the conversion of glutamate to proline (Weber, 1965; Weber and Ogawa, 1965).

Different fungal spores may have varying capacity to synthesize amino acids during germination. Staples *et al.* (1961) observed that in parasitic fungi like *Uromyces phaseoli*, the amino acid synthesis in germinating spores occurred rather slowly as compared to saprophytic ones. Moreover, the spectrum of amino acids synthesized by saprophytes was also wider than that of the obligate parasites.

*Synthesis of proteins.* Most of the biosynthetic activities of a germinating spore are ultimately meant for protein synthesis, which is so essential for the formation of new protoplasm. The endogenous pool of amino acids finally contribute to the synthesis of protein macromolecules. Investigations by Shepherd (1957) as well as Yanagita and others (Yanagita, 1957; Takabe and Yanagita, 1959) have shown that amino acids required by the spores of *Aspergillus* spp. are utilized and transformed immediately into a pool of soluble amino acids, which later contribute to proteins. However, some other nitrogen compounds may also contribute to protein synthesis, but they allow very little protein-synthesizing activity during the first one third (or so) of the time required for germination (Shepherd, 1957). Nevertheless, the total protein content was found to get doubled prior to

emergence of the germ-tube, during which the amino acid pool also declined (Yanagita 1957; Shepherd, 1957; Lovett and Cantino, 1960). Similar increase in the protein content was noted in the germinating conidia of *Penicillium oxalicum*, *P. atrovirens*, *Aspergillus niger* and *Trichoderma viride* by Ohmori and Gottlieb (1965) in the ascospores of *Neurospora tetrasperma* by Hill and Sussman (1964); and in conidia of *Botryodiplodia theobromae* (Etten *et al.* 1972). In fact, protein-synthesis has been shown to occur in most of the fungal spores studied so far.

Rate of protein-synthesis during spore germination seems to vary in different fungi, and under different conditions of incubation. Shu *et al.* (1954) as well as Staples *et al.* (1961, 1962) noted such differences between the spores of some saprophytic fungi and some rusts. They observed that spores of *Glomerella cingulata*, *Neurospora sitophila* and *Aspergillus niger* readily synthesized protein during germination, but in spores of *Puccinia graminis tritici*, *P. helianthi*, *P. sorghi* and *Uromyces phaseoli* synthesis of protein did not take place. In fact, uredospores of *P. graminis tritici* have been reported to show a decline in their protein-content during germination (Shu *et al.* 1954).

Available evidences indicate that the biosynthetic pathway of protein formation in spores follows more or less the same course as is known for fungal mycelium, or that operating in the animal and bacterial cells (discussed in Chapter X). The activated amino acid reacts with the soluble ribonucleic acid (sRNA) to form the aminoacyl-sRNA, which is transferred to the ribosomes for the formation of peptides and then to the complete protein macromolecule. Evidences to support the operation of such a pathway in fungal spores have primarily been derived from the inhibiting action of certain antibiotics known to affect protein synthesis (Niederpruem, 1964) and of some respiratory poisons like cyanide and azide, but it should be emphasised that these are only indirect evidences. Among the very few direct evidences the one noted by Henney and Storck (1964) may be cited. They have demonstrated the occurrence of polyribosomes in extract from germinated spores of *Neurospora*. Obviously, more direct investigations are awaited.

*Synthesis of nucleic acids and nucleoproteins.* Germination of spore in many fungi is marked by one or more nuclear division during the enlargement phase. It is obvious therefore, that synthesis of nuclear components takes place in such spores, before the onset of germination. Similarly, specific RNA, like soluble RNA involved in protein.

synthesis must also be formed prior to initiation of protein synthesis. This is not to suggest that these components are entirely lacking in resting spores, rather most of the basic requirements for germination are present in a spore, but in a limited quantity, and hence their amount must be increased to meet the requirement of germination.

Synthesis of both RNA and DNA is initiated early in the germinating conidia of *Aspergillus niger* and attains maximum proportions during the first six hours (Yanagita, 1956, 1957). In a later study, Yanagita (1963) recorded that nucleic acids constituted about 10% of the total carbon content of these conidia and that RNA synthesis was initiated still earlier *i.e.* within 30 minutes, while DNA synthesis started only after 3 hours of incubation. In *Aspergillus nidulans* also RNA synthesis preceded DNA formation, but after five hours of incubation, synthesis of both the nucleic acids were equal. Turian and Cantino (1959), tried to correlate the nucleic acid content of *Blastocladia* zoospores with the number of nuclei present. Data obtained by them indicate that during nuclear division DNA is synthesized at the expense of RNA, which remains concentrated in the spores of this fungus in the form of nuclear caps. Yanagita (1957) noted that RNA synthesis in *A. niger* conidia proceeded in phases, but during germination RNA/DNA ratio declined markedly, which possibly indicated a rapid synthesis of DNA. However, it must be noted that any appreciable change in DNA content may only be expected when there is a formation of new nuclei, or of nuclear material for maturation of nuclei, which normally occurs late in the germination process and is often considered a post-germination phenomenon. Dunkle *et al.* (1972) found that DNA-synthesis was not required for spore germination of *Botryodiplodia theobromae*, and was essential for continued vegetative growth. Unfortunately, there has been little attempt to correlate the DNA content of germinating spores with the cytological changes occurring in them, and the few available reports do not form a comprehensive picture. In some of the spores, the DNA content has been reported to increase during germination, which may possibly indicate that formation of nuclear materials may take place prior to cell-formation. In others, DNA-content has been reported to remain almost static during germination (Nishi, 1961) or atleast during the early part of this process (Shepard, 1957; Yanagita, 1963), which indicates the contrary. However, there are sufficient data (cf. Gottlieb, 1965) to suggest that wherever DNA synthesis takes place during germination, it starts much after the initiation of RNA synthesis and is far exceeded by the latter.

*Synthesis of wall materials.* A germinating spore accomplishes the synthesis of various wall-components before it protrudes its germ-tube. The uredospores of rust fungi were found to increase in their chitin (Shu *et al.* 1954) and glucosamine (Staples *et al.* 1962) contents during germination. Evidences for synthesis of different wall materials during germination have also been obtained from *Aspergillus nidulans* (Shepard, 1956), *A. niger* (Hoshino, 1961) and *Fusarium solani* f. *phaseoli* (Cochrane *et al.* 1963 c). In fact, in some spores the wall materials are laid in the form of a third layer of spore-wall, before the emergence of the germ-tube. Obviously, this third layer later forms the germ-tube wall. Electron microscopic studies on various fungal spores, including those of *Rhizopus* spp. (Hawker and Abbott, 1963) *Cunninghamella elegans* (Hawker, 1966) and *Gilbertella persicaria* (C.E. Bracker, cited by Gottlieb, 1966), have shown the formation of a new wall-layer, which subsequently envelops the emerging germ-tube. However, in spores of some other fungi, like *Botrytis cinerea*, *Penicillium frequentans* and *Byssoschlamys fulva* possibly no new wall layer is laid during germination (Hawker, 1966). However, the question is still not fully resolved because the origin of the two wall layers visible in the germinating spores of *B. cinerea* is not yet fully established. Although, further ultra-structural studies both during sporulation as well as germination may be helpful in this regard, yet the question could have been better tackled at the biosynthetic level, because studies relating to the synthesis of wall-components prior to germ-tube formation may throw sufficient light on this problem.

## FUNGAL METABOLITES

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Fungi are well known for their chemosynthetic abilities and produce a wide variety of organic compounds, including a large number of such substances, whose function for the producing organism is still obscure. Such compounds are considered as secondary metabolites. As per the current concept, secondary metabolites of fungi are defined as those natural products of fungal metabolism, (i) which possess no obvious function in cell-growth; (ii) which are synthesized by cells that have stopped dividing and (iii) which show specific taxonomic distribution. Thus, this class of compounds does not include substances which are synthesized by dividing cells and which are grouped as primary metabolites. Emphasis in this chapter will be on secondary metabolites, but a few important primary ones are also included.

Fungal products had attracted the earlier mycologists and chemists but a systematic approach to this group of substances was actually initiated by Raistrick and his associates who at the instance of Sir Frederick Gowland Hopkins, started their classic investigations on chemical activities of fungi (Raistrick, 1931). Their discovery of a good number of new and interesting fungal metabolites and subsequent discovery of penicillin by Flemming led to an intense screening of fungi for isolation of newer antibiotics or other pharmacologically important products. The rapid advances made in this field of investigation have been more remarkable since 1956 onward owing mainly to the application of isotopic technique in these studies. To-day, not only a large number of fungal metabolites with antibiotic, toxic, and growth promoting properties are known to science but a considerable understanding of the chemical structure and biogenesis of these metabolites has been achieved. Investigations into the biosynthetic pathways of the secondary metabolites have further made it clear that the metabolic activities resulting in this class of

compounds though designated as secondary, they are no less important and they can very well contribute to our understanding of the microbial biology (Bu'Lock, 1967). A vast literature on fungal metabolites has accumulated, which has been reviewed by Shibata *et al.* (1964), Birkinshaw (1965), Whalley (1967), Weinberg (1970) and Turner (1971).

As indicated earlier, secondary metabolites are synthesized rather late, subsequent to cessation of cellular multiplication, and after certain period their synthesis stops, although the cells may continue to be viable. In filamentous fungi, small amounts of secondary metabolites appear, while the culture is still showing increase in contents of nucleic acids and protein. It is believed that it is due to those cells of the filament, which have stopped dividing. Otherwise it is quite certain, that bulk of the secondary metabolites are formed, after the increase in the nucleic acid, protein and cell number has come to a halt. Another characteristic feature of secondary metabolites is their high degree of specificity, and are thus produced either by a single species or very few species. Despite such extra-ordinary specificity these substances show great chemical diversity, and are found in a variety of chemical groups of compounds, some of which are tabulated here:

TABLE 16.1

SHOWING VARIOUS CLASSES OF ORGANIC COMPOUNDS IN WHICH  
SECONDARY METABOLITES OF FUNGI AND BACTERIA ARE  
FOUND ASSOCIATED

Amine sugars	Lactones	Pyridines
Anthocyanins	Macrolides	Pyrones
Anthraquinones	Naphthalenes	Pyrroles
Aziridines	Naphthaquinones	Pyrrolines
Benzoquinones	Nucleosides	Pyrrolizines
Coumarins	Oligopeptides	Quinolines
Diazines	Perylenes	Quinololins
Epoxides	Phenazines	Quinones
Ergoline alkaloids	Phenoxazinones	Salicylates
Flavonoids	Phthaldehydes	Terpenoids
Glutamides	Piperazines	Tetracyclines
Glycosides	Polyacetylenes	Tetronic acids
Hydroxylamines	Polyenes	Triazines
Indole derivatives	Pyrazines	Tropolones



From the listed compounds in Table 16.1, wide range of chemical structure possible for secondary metabolites is clear. Despite this diversity, a large number of families of secondary metabolites are synthesized from similar and simpler precursors. Generally, primary metabolites like acetate, pyruvate, malonate, mevalonate, shikimate, prephenate, amino acids and purines, or transient or intermediate secondary metabolites such as orsellinic acid and 6-methylsalicylic acid (Bulock *et al.* 1965) act as the precursor units for the biosynthesis of secondary metabolites.

Formation of secondary metabolites does not seem to be connected with the basic biosynthetic capabilities of biomass formation. Many a times these metabolites are produced in large quantities, although they are of little significance to the growth phase of the micro-organisms. Towards the end of logarithmic phase, marked changes take place in the biosynthetic activities. It has been observed that during this transitory stage, subsidiary metabolic pathways become the main metabolic routes, replacing those activities which were till then the principal metabolic streams and were contributing to the synthesis of cell-mass and cell-walls of the proliferating cells. Thus, the stationary phase of growth, also called idiophase is marked by the predominance of secondary metabolic activities. Other features of this phase are reduced respiratory activity (Hawker, 1960) and growth rate but rapid phenol synthesis. Changes in the biosynthetic activities during the idiophase clearly indicate that certain enzymes are synthesized *de novo* and/or those formed during trophophase (=logarithmic phase) and were lying suppressed under catabolite repression are unmasked. Biosynthetic changes have been correlated with transition of growth phases in a number of fungi. *Penicillium urticae* does not synthesize or metabolize 6-methylsalicylic acid during trophophase but as soon as the growth phase advances into idiophase, 6-methylsalicylic acid appears in the culture. Similar reports for various other metabolites are also available (Borrow *et al.* 1961; Rhodes *et al.*, 1963).

Nevertheless, biosynthesis of these metabolites involves rather limited number of reactions, interlinked with the general metabolism of the organism (Fig. 16.1).

It appears that there are two main pathways, during which most of the fungal metabolites are synthesized: (i) the acetate pathway

and (ii) the shikimic acid pathway. Elucidation of both these pathways has been greatly aided by (a) use of mutant strains of fungi lacking some specific enzyme catalyzing a particular reaction of the

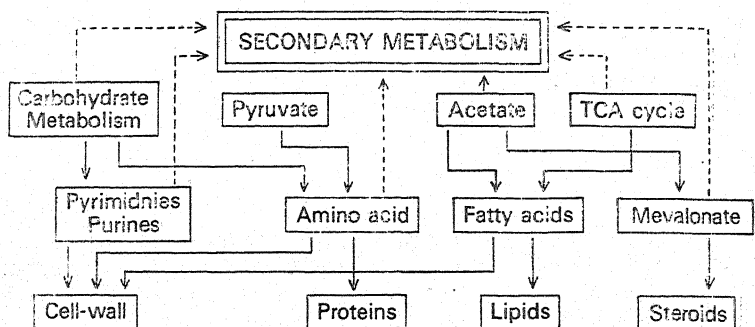


Fig. 16.1. Interrelations of various metabolic Pathways.

pathway and (b) use of substrate labelled with radioactive isotopes, generally  $^{14}\text{C}$ , followed by use of suitable chemical methods to determine the fate of individual atoms of the substrate during the biosynthetic pathway.

### ACETATE PATHWAY

It is postulated that during this pathway there is a "head-to-tail" union of acetate units, resulting in the biosynthesis of a large number of fungal metabolites, particularly in the aromatic series. The idea was first conceived in a very simple manner by Collie in 1893, and some time later it was repeated by Birch and Donovan (1953) as well as Robinson (1955). The latter suggested that the biosynthesis of endocrocin, a metabolite of *Aspergillus amstelodami* could involve a head-to-tail linkage of eight acetate units to form a polyketo acid of 16 carbon atoms, which on reduction could form palmitic acid. However, by the use of appropriate ring closures, this chain could be folded into a ring form, very much identical with the structure of anthrone corresponding to endocrocin. Further, it was presumed that the anthrone form is readily oxidized into the anthroquinone form of endocrocin.

Experimental support to all these presumptions was first provided by Birch and his associates who with a series of careful experiments

employing acetates labelled with isotopic carbon  $^{14}\text{C}$  were able to establish this pathway. Birch *et al.* (1955) grew *Penicillium griseofulvum* on a glucose medium containing  $\text{CH}_3^{14}\text{COOH}$ , and then examined the position and degree of labelling in 2-hydroxy-6-methyl benzoic acid produced by the fungus. The pattern of labelling was

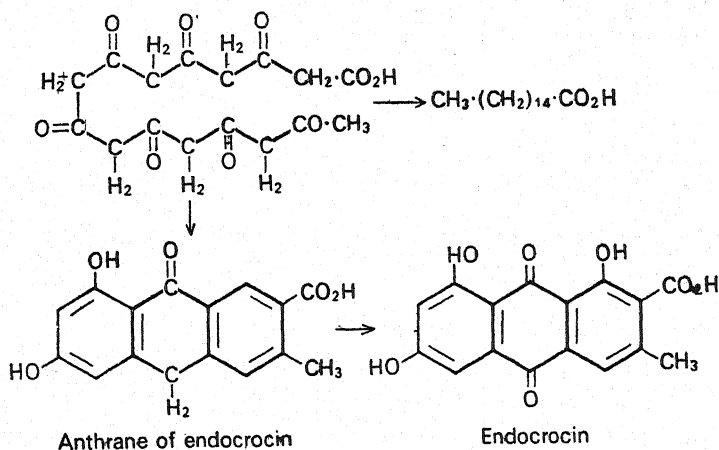
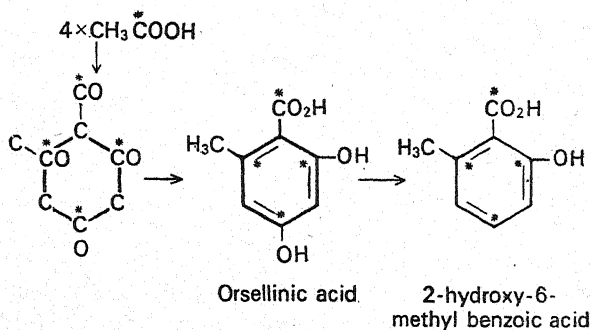


Fig. 16.2. Biosynthesis of endocrocin.

found to be in complete accord with their hypothesis that the 6-methyl benzoic acid molecule originates from a chain of four acetate units linked head-to-tail, as shown hereunder:

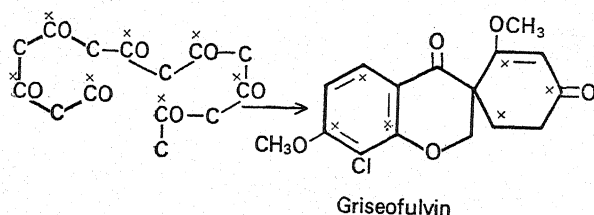


Orsellinic acid, which is in itself a secondary metabolite is considered to act as an intermediate product giving rise to 6-methyl benzoic

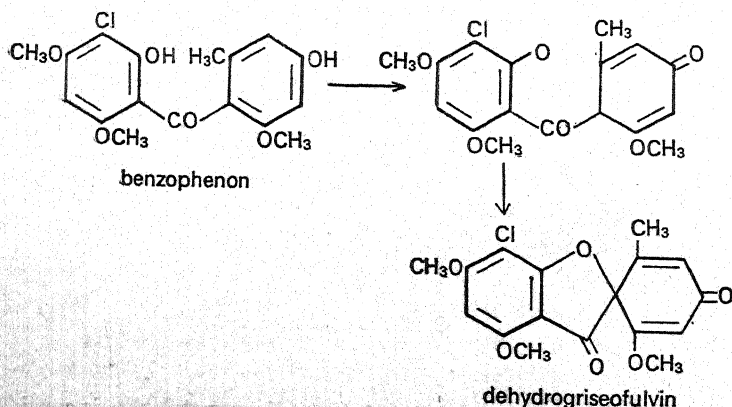
acid after undergoing a reduction step to eliminate a hydroxyl group. Biosynthesis of orsellinic acid, which is a metabolite of *Chaetomium cochlioides*, has also been shown to acquire similar labeling pattern when grown on labelled acetate (Gatenbeck and Mosbach, 1959).

Biosynthesis of some other fungal metabolites involving head-to-tail union of acetate units which have been studied with labelled substances are described below:

**Griseofulvin.** It is formed by *Penicillium griseofulvum*, and its biosynthesis involves the union of seven acetate units:



Evidences indicate that in the penultimate step of griseofulvin biosynthesis, dehydrogriseofulvin is formed, the hydrogenation of which gives rise to griseofulvin. The steps are shown below:



Two metabolites of *Aspergillus terreus*, viz. geodin and erdin show close structural similarity with griseofulvin, and possibly they are also synthesized via a similar route. In fact, the metabolites of *A. terreus* also contain geodoxin, and asteric acid. Asteric acid, in turn may be chemically synthesized from yet another fungal metabolite sulochrin (Curtis *et al.* 1960) produced by *Oospora sulphurea-ochracea*, as well as *Penicillium frequentans* (Stickings and Mahmoodian 1962). All these evidences indicate that geodin, erdin, geodoxin, asteric acid and sulochrin may under natural conditions be interconvertible (Whalley, 1967).

**Alternariol.** This metabolite is produced by *Alternaria tenuis* and its biosynthesis involves seven acetate units:

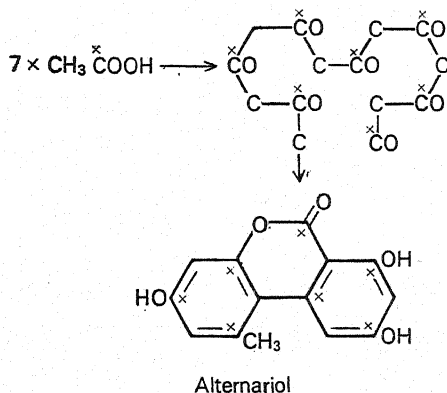


Fig. 16.3. Structure of Alternariol.

**Atrovenetin.** This is a metabolite of *Penicillium atrovenetum*. The nucleus of this compound is derived from seven acetate units.

Isotopic studies on *Penicillium atrovenetum* have also indicated that the perinaphthenone nucleus of atrovenetin is derived from acetate units and the side-chain at C<sub>5</sub> from acetate or mevalonate. The sequence of the biosynthesis has been suggested as follows (Thomas, 1961):

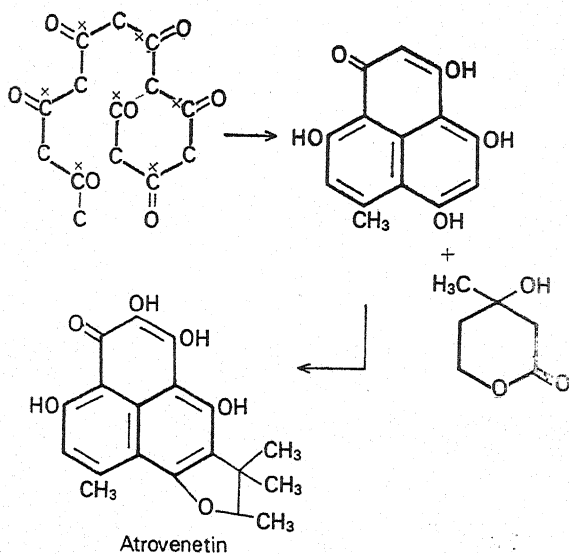


Fig. 16.4. Biosynthesis of atrovenetin.

*Curvularian*. Derived from eight acetate units, curvularin is a metabolite of *Curvularia species* (Birch *et al.* 1959). This compound has an interesting chemical structure and is a naturally occurring macrolide lactone, with a methyl group as the only alkyl substituent. It is considered to provide a biosynthetic link between macrolide antibiotics and phenols derived from acetate. The structure of curvularin as well as the pattern of isotopic labeling is shown below:

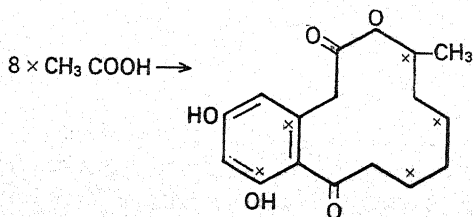


Fig. 16.4 (a) Curvularin.

These are the few examples, which have provided isotopic evidences for the operation of acetate pathway during the biosynthesis of fungal products. However, recent researches have indicated that

during the head-to-tail union free acetate units do not take part, rather acetyl CoA units are involved, and the resulting chain contain a terminal thiol ester group. Therefore, free metabolite is obtained after the thiol ester is hydrolysed. Bu'Lock and Smalley (1961) have shown that during the biosynthesis of 6-methylsalicylic acid, three out of 4 acetate units are in the form of malonate, probably as molo-nyl coenzyme A. They consider that the three malonate units subsequently lose three molecules of  $\text{CO}_2$  and give rise to the metabolite. The overall effect obviously remains the same, which would have been produced by the union of 4-acetate units. The fungus, if not supplied with malonate, is able to synthesize it from acetyl CoA and carbon dioxide by carboxylation. Biosynthesis of penicillic acid by *Penicillium cyclopium* also involves the participation of malonate. The following sequence of reactions for penicillic acid synthesis was proposed by Bentley and Keil (1961):

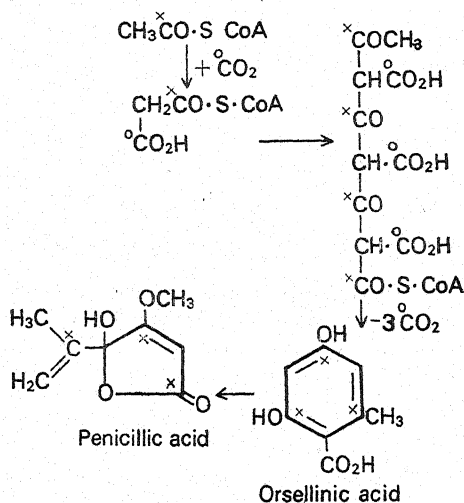
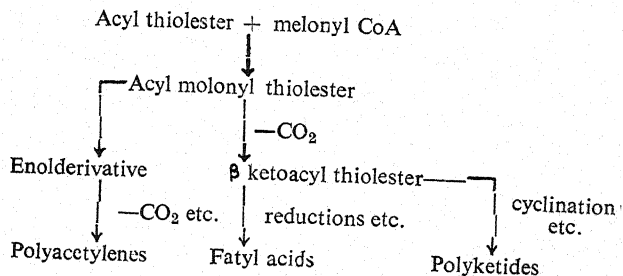


Fig. 16.5. Biosynthesis of penicillic acid.

This has necessitated a slight modification in the acetate theory, and it is now believed that the first (or "starter") unit in any polyketomethylene system must be a monobasic acid, like acetic acid, while the subsequent units are derived from malonate, or sometimes methyl malonate. These modifications were suggested on the basis of data



obtained from isotopic studies on *P. urticae*, which produces 6-methyl salicylic acid as a metabolite. When mycelium of this fungus was resuspended in a glucose-free medium supplemented with labelled sodium acetate ( $^{14}\text{C}_1$ ) and unlabelled malonate, the latter was utilized in preference over labelled acetate for the formation of the entire carbon of the 6-methylsalicylic acid, except the terminal ( $\text{C}_6$ ) carbon containing methyl group, which obviously was derived from the labelled acetate (Birch *et al.* 1961; Bu'Lock and Smalley, 1961). Malonate seems to contribute to biosynthesis of polyacetylenes also (Bu'Lock and Smalley, 1962) besides its known contribution to fatty acid synthesis. These authors have proposed a common scheme through which acetyl, thiolester and malonyl CoA might be contributing to the formation of polyacetylenes, fatty acids as well as the acetate-derived aromatics, the polyketides:



However, little is known of the intermediate steps of this scheme, which clearly needs further elucidation.

### SHIKIMIC ACID PATHWAY

Although most of the aromatic metabolites of fungi are synthesized by way of the acetate pathway, some of them are derived through a sequence of reactions in which shikimic acid or its biogenetic associates play a major role. The pathway is, therefore, rightly designated as the shikimic acid pathway. Many of the steps of this pathway could be elucidated as a result of studies on some aromatic amino-acid-deficient mutants of *Neurospora crassa*, besides a few bacteria. The concerning literature has been reviewed by Davis (1955).

The initial substrates of this pathway are phosphoenol pyruvic acid and D-erythrose 4-phosphate, derived from EM and PP' pathways.

of glucose catabolism, respectively. A condensation reaction between the two gives rise to 2-keto-3-deoxy-D araboheptonic acid 7-phosphate, which by some rearrangement and condensation leads to the formation of shikimic acid. Importance of shikimic acid is due to its role in the synthesis of aromatic amino acids, like phenylalanine and tyrosine. The shikimic acid pathway is illustrated in Fig. 16.3.

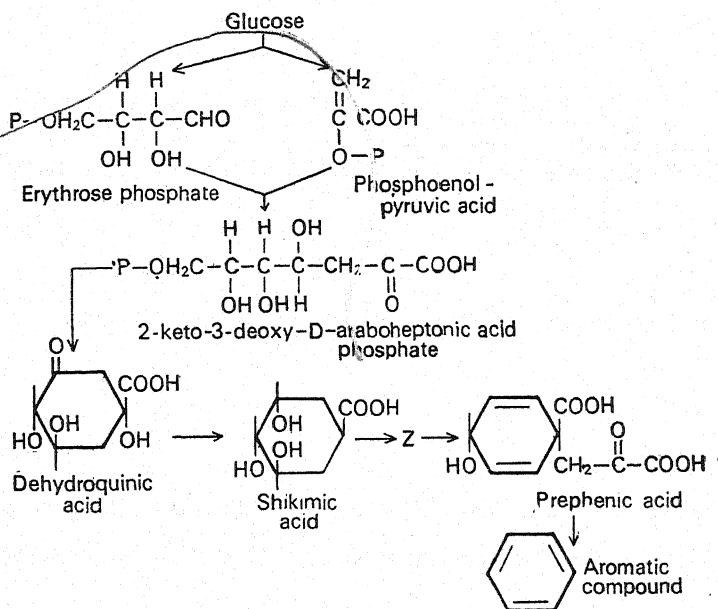


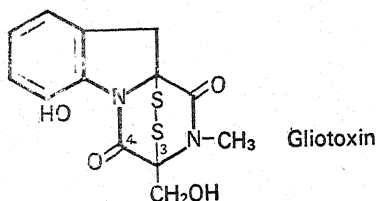
Fig. 16.6. Shikimic acid pathway.

As shown above, the shikimic acid condenses with a 3-carbon unit to form an unknown intermediate 'Z', which transforms into prephenic acid. Prephenic acid marks the stage where aromatization takes place, giving rise either to tyrosine through p-hydroxy phenyl pyruvic acid, or to phenylalanine through phenylpyruvic acid. Anthranilic acid and tryptophane are also derived from shikimic acid.

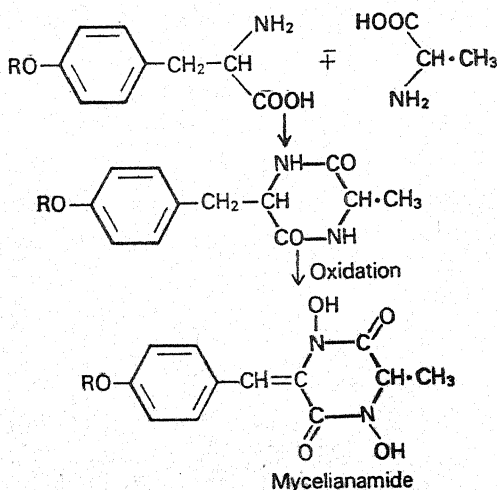
Evidences obtained from isotopic experiments indicate that these aromatic amino acids are incorporated *en bloc* into the aromatic rings of the resultant metabolite. When *Trichoderma viride* was

grown with  $^{14}\text{C}_1$ -phenylalanine or phenylalanine- $\text{H}^3$  (Winstead and

Suhadolnik, 1960), it was observed that the entire amino acid molecule was incorporated into the indole ring of gliotoxin, a sulphur containing metabolite of the fungus. It appears that carbon atoms 3, 3a and 4 are contributed by serine, while the N-methyl residue is derived from methionine.



Similarly, tyrosin (obviously through shikimic acid) and alanine contribute in the formation of benzenoid ring of mycelianamide, a primary metabolite of *Penicillium griseofulvum*. The possible steps in the incorporation of tyrosine and alanine are shown below:



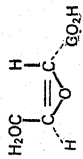
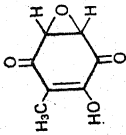

Biosynthesis of various other aromatic metabolites of fungi, like benzoic acid, cinnamic acid, anisaldehyde, anisic acid, gallic acid, volucrisporin, etc. also takes place through shikimic acid pathway. Some such products alongwith their sources have been shown in Table 16.2; which also incorporates some biosynthates of acetate

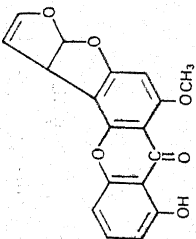
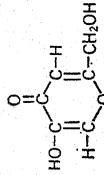
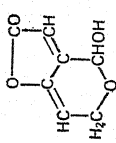
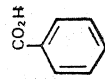
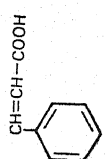
TABLE 16.2  
FUNGAL METABOLITES


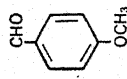
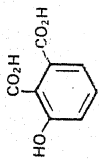
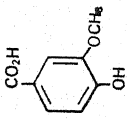
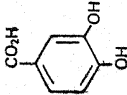
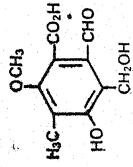
Chemical Groups	Metabolite	Structure	Source/s	Author/s
1	2	3	4	5
I. ALIPHATIC SATURATED COMPOUNDS				
Esters	Ethyl acetate	$\text{CH}_3\text{CO}_2\text{C}_2\text{H}_5$	<i>Penicillium digitatum</i>	Birkinshaw <i>et al.</i> (1931)
"	Isobutyl acetate	$\text{CH}_3\text{CO}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_3$	<i>Endoconidiophora coerulescens</i>	Birkinshaw and Morgan (1950)
Acids	$\alpha$ -Methylbutyric acid	$\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3)\text{CO}_2\text{H}$	<i>Penicillium notatum</i>	Cram and Tishler (1940)
"	Dimethyl pyruvic acid	$\text{CH}_3\text{CH}(\text{CH}_3)\text{COCO}_2\text{H}$	<i>Aspergillus</i> species	Ramchandran and Radha (1955)
"	$\alpha$ , $\beta$ -dihydroxy- $\beta$ -methyl valeric and $\alpha$ , $\beta$ -dihydroxyisovaleric acids	$\text{CH}_3\text{CH}_2\text{C}(\text{OH})(\text{CH}_3)\text{CH}(\text{OH})\text{CO}_2\text{H}$ $\text{CH}_3\text{C}(\text{OH})(\text{CH}_3)\text{CH}(\text{OH})\text{CO}_2\text{H}$	} <i>Neurospora crassa</i>	Sjolander <i>et al.</i> (1954)
"	Spiculisporic acid (hydrate)	$\text{CH}_3(\text{CH}_2)_9\text{CH}(\text{CO}_2\text{H})\text{C}(\text{OH})\text{CO}_2\text{HCH}_2\text{CH}_2(\text{CO}_2\text{H})$		Clutterbuck <i>et al.</i> (1931) and Asano and Kameda (1941)
"	Minoliuteic acid (hydrate)	$\text{CH}_3(\text{CH}_2)_{13}\text{CH}(\text{CO}_2\text{H})\text{C}(\text{OH})\text{CO}_2\text{H}\cdot\text{CH}_2(\text{CO}_2\text{H})$	<i>P. minioluteum</i>	Birkinshaw and Raistrick (1934)
"	Agaric acid	$\text{CH}_3(\text{CH}_2)_{15}\text{CH}(\text{CO}_2\text{H})\text{C}(\text{OH})\text{CO}_2\text{H}\cdot\text{CH}_2(\text{CO}_2\text{H})$	<i>Fomes officinalis</i>	Thoms and Vogelsang (1907)
"	Caperatic acid	$\text{CH}_3(\text{CH}_2)_{13}\text{CH}(\text{CO}_2\text{H})\text{C}(\text{OH})\text{CO}_2\text{H}\cdot\text{CH}_2(\text{CO}_2\text{H})$	<i>Parmelia caperata</i>	Asano and Ohta (1933, 34)
Alcohols	Cetyl alcohol	$\text{CH}_3(\text{CH}_2)_{14}\text{CH}_2\text{OH}$	<i>Amanita phalloides</i>	H. Wieland and Coutelle (1941)
"	Stearyl alcohol	$\text{CH}_3(\text{CH}_2)_{16}\text{CH}_2\text{OH}$	<i>Penicillium notatum</i>	Angeletti <i>et al.</i> (1952)
Hydrocarbon	Octacosane	$\text{CH}_3(\text{CH}_2)_{26}\text{CH}_3$	<i>Amanita phalloides</i>	H. Wieland and Coutelle (1941)

1	2	3	4	5
II. ALIPHATIC UNSATURATED COMPOUNDS				
Hydrocarbons	Ethylene	$\text{CH}_2=\text{CH}_2$		
"	Trimethylethylene	$\text{CH}_3\text{C}(\text{CH}_3)=\text{CHCH}_3$	<i>Penicillium digitatum</i> <i>Fusarium oxysporum</i> f. <i>lycopersici</i> <i>Puccinia graminis</i> f. sp. <i>tritici</i>	Hall (1951) Dimond and Waggoner (1953) Forsyth (1955)
" (Polyenyne)	Agrocybin	$\text{HOCH}_2-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CONH}_2$	<i>Agrocybe dura</i>	Anchel (1952), Bu'Lock <i>et al.</i> (1954)
" (Polyenyne)	Diatretynes	$\text{HO}_2\text{C}-\text{CH}=\text{CH}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{R}$	<i>Clitocybe diatreta</i>	Anchel (1953, 1955) Ashworth <i>et al.</i> (1958)
" (Polyacetylene)	Alcohol	$\text{CH}_3-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CH}=\text{CH}-\text{CH}_2\text{OH}$	<i>Pleurotus ulmarius</i> <i>Poria sinuosa</i>	(cf. Birkinshaw, 1965) Cambine <i>et al.</i> 1963

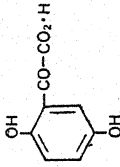
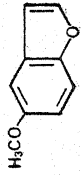
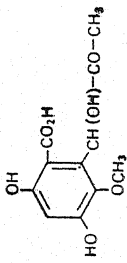
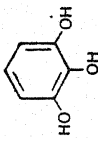
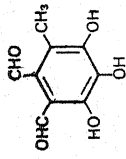
### OXYGEN HETEROCYCLES

Epoxides	Epoxysuccinic acid		<i>Aspergillus fumigatus</i>	Sakaguchi <i>et al.</i> (1939) Birkinshaw <i>et al.</i> (1945)
"	Terreic acid		<i>A. terreus</i>	Flory <i>et al.</i> (1949) Sheehan <i>et al.</i> (1958)
Furans	2-Hydroxymethylfuran-5-Carboxylic acid		<i>A. niger</i>	Sumiki (1931)

1	2	3	4	5
"	Sterigmatocystin		<i>A. versicolor</i>	Hatsuda and Kuyama (1954) Bullock <i>et al.</i> (1962)
Pyrones	Kojic acid		<i>A. flavus, A. oryzae</i>	Yobuta (1924)
"	Patulin		<i>Penicillium patulum</i>	Birkinshaw <i>et al.</i> (1943 a) Woodward and Singh (1949)
III. AROMATIC MONOCYCLIC COMPOUNDS				
Substituted Benzenes	Benzoic acid		<i>Penicillium roseopurpureum</i>	Posternak (1940)
"	Cinnamic acid		<i>Stereum subpileatum</i>	

1	2	3	4	5
"	Methyl cinnamate		<i>Leninus lepidus</i>	Birkinshaw and Findlay (1940)
"	Anisaldehyde		<i>Daedalea Juniperina</i> <i>Polyporus benzoinus</i>	Birkinshaw and Chaplen (1955) Birkinshaw et al. (1952)
"	3-Hydroxyphthalic acid		<i>Penicillium islandicum</i> , <i>P. patulum</i>	Gatenbeck (1957)
"	Vanillic acid		<i>Neurospora crassa</i>	Metzenberg and Mitchel (1958)
"	Protocatechuic acid		"	"
"	Cyclopolic acid		<i>Penicillium cyclopium</i>	Birkinshaw et al. (1952 c)



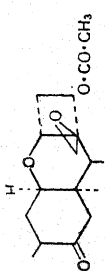
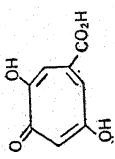
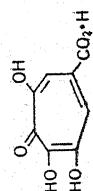
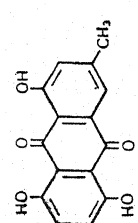
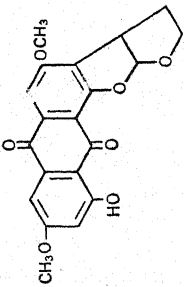
1	2	3	4	5
Substituted Benzenes	2, 5-Dihydroxyphenyl-glyoxylic acid		<i>Polyporus tumulosus</i>	Moir and Ralph (1954)
"	5-Methoxycoumarone		<i>Stereum subpileatum</i>	Birkinshaw <i>et al.</i> (1957)
"	Ustic acid		<i>Aspergillus ustus</i>	Raistrick and Stickings (1951)
"	Pyrogallol		<i>Penicillium patulum</i>	Bassett and Tanenbaum (1958)
"	Flavipin		<i>Aspergillus flavipes</i>	Raistrick and Rudman (1956)

1	2	3	4	5
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#### IV. AROMATIC POLYCYCLIC COMPOUNDS

Asterric acid		<i>Aspergillus terreus</i>	R.F. Curtis <i>et al.</i> (1960)
Citrinin		<i>Penicillium citrinin</i> <i>Aspergillus terreus</i>	Hetherington and Raistrick (1931) Johnson <i>et al.</i> (1950)
Isocoumarin derivative		<i>Aspergillus terreus</i>	Hassall and Jones (1962)
Gibberellic acid		<i>Gibberella fujikuroi</i>	P.J. Curtis and Cross (1954) Aldridge <i>et al.</i> (1963)
Verrucarol		<i>Myrothecium verrucaria</i>	Tamm and Gutzwillen (1962)

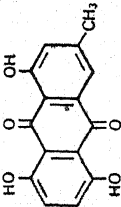
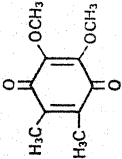
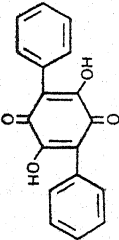
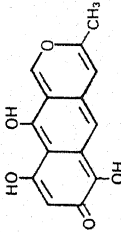
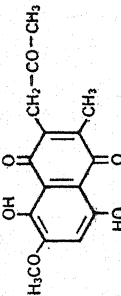
#### TERPENES

1	2	3	4	5
	Trichodermin		<i>Trichoderma</i> sp.	Godfredsen and Vangedal (1964) Abrahamsson and Nilsson (1964)
	Stipitatic acid		<i>Penicillium stipitatum</i>	Birkinshaw <i>et al.</i> (1942 c) Corbett <i>et al.</i> (1950 c)
	Puberulic acid		<i>Penicillium puberulum</i>	Birkinshaw and Raistrick (1932) Corbett <i>et al.</i> (1950 a, b)
	Averufin		<i>Aspergillus versicolor</i>	Bullock <i>et al.</i> (1963)
	Aversin		"	Pusey and Roberts (1963)
	"			

## TROPOLONES

## QUINONES

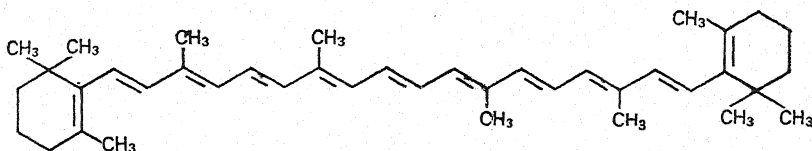
## Anthraquinone

1	2	3	4	5
	Helminthosporin		<i>Helminthosporium gramineum</i>	Charles <i>et al.</i> (1933), Birch <i>et al.</i> (1958)
<i>Benzoquinones</i>	Aurantio-glucoladin		<i>Glucoladium</i> sp.	Vischer (1953)
	Polyporic acid		<i>Polyporus nidulans</i>	Kogl (1926)
<i>Hemiquinones</i>	Purpurogenone		<i>Penicillium purpurogenum</i>	Roberts and Warren (1955)
<i>Naphthoquinones</i>	Javanicin		<i>Fusarium javanicum</i>	Arnstein and Cook (1947) Ruelius and Gauhe (1950)

pathway. A general description of the classes of compounds, synthesized by fungi through their primary and/or secondary metabolic activities is briefly summarised below, alongwith the biosynthesis of a few important ones.

## Aliphatic Compounds

Many saturated and unsaturated aliphatic metabolites including acids, alcohols, esters, polyacetylene etc. are synthesized by fungi, a few of them are shown in Table 16.2. Some of the metabolites show both saturated single bonds and unsaturated double bonds in their molecule. Good examples of such kinds of metabolites are the carotenoid, which are common constituent of fungi. The yellow pigment,  $\beta$ -carotene has the following chain-structure:

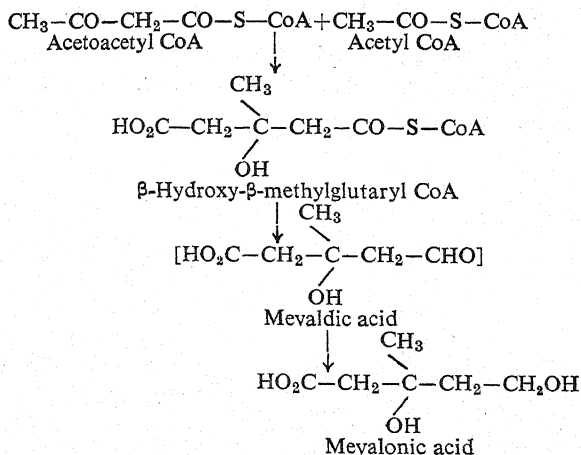


### Structure of $\beta$ -Carotene

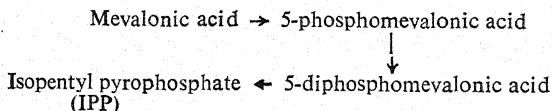
**Fig. 16.7.** Structure of  $\beta$ -carotene.

It may be noted that the molecular skeleton is composed of eight isoprene ( $\text{CH}_2=\text{CH}_3\text{C}-\text{CH}=\text{CH}_2$ ) units, and is symmetrical with respect to the terminal rings.

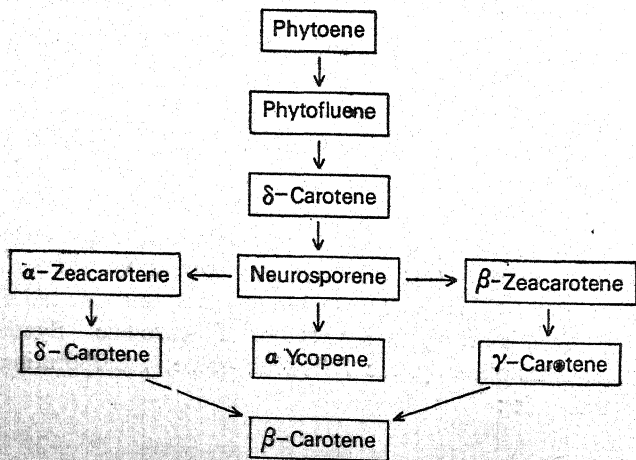
Synthesis of  $\beta$ -carotene has mostly been studied in Mucorales and yeasts. Experiments with radioactive acetate (Grob *et al.* 1951, 1956) as well as labeled mevalonate (Grob, 1957; Braithwaite and Goodwin, 1960) have shown that these compounds are incorporated into  $\beta$ -carotene in *Mucor hiemalis* *Phycomyces blakesleeanus* etc. Mevalonic acid which is known to produce isoprene units by its decarboxylation, is the obvious precursor of  $\beta$ -carotene. Synthesis of mevalonate takes place by the following route, incorporating in all three acetate units:



Next important intermediate in the synthesis of carotenoids is isopentyl pyrophosphate (IPP) which is possibly formed as under:



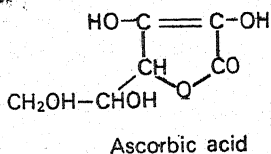
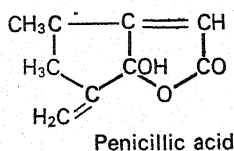
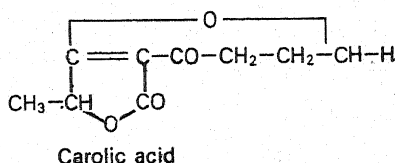
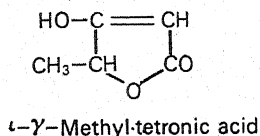
The IPP subsequently condenses with its isomer dimethylallyl pyrophosphate (which is produced from IPP, by an isomerase enzyme to form a 10-carbon compound Geranyl PP. Several condensation steps ultimately lead to the formation of a skeleton of 40 carbon



atoms which functions as the primary unit. The chemical nature of the primary unit is not yet confirmed, although with the limited information available phytoene seems to be the probable contender (Burnett, 1968). Beyond phytoene the probable steps of carotenogenesis seem to follow.

### Oxygen Heterocycles

Different kinds of oxygen-heterocyclic metabolites are known to be produced by fungi, several of which have been illustrated in Table 16.2. Some important tetronic acids produced by fungi are being discussed here. Tetronic acids have a furanoid structure and are acidic even though some of them may not have a free carboxyl group, *e.g.*, ascorbic acid. Some of the representative types of this group are represented below:



Till now *Penicillia* have proved to be a good source of tetronic acids, and attempts to screen other fungal genera for these metabolites may seem interesting.

The various tetronic acids seem to be synthesized by different biosynthetic routes. Lybing and Reio (1958) on the basis of isotopic data concluded that carlosic acid is derived partly from acetyl units and partly from a dicarboxylic acids of TCA cycle. Ascorbic acid biosynthesis, however, is known to occur during glucuronate-xylulose pathway, from D-glucuronic acid and through L-gulonolactone and 2-keto-L-gulonolactone (Chapter VII). Similarly, synthesis of penicillic acid follows yet another sequence and is produced by the cleavage of the aromatic ring of orsellinic acid.



### Aromatic metabolites

These fungal products are characterized by an aromatic ring of six carbon atoms and with three unsaturated double bonds. There may be one or more than one such rings in an aromatic compound, and accordingly they are grouped as monocyclic or polycyclic. However most of the monocyclic aromatic metabolites of fungi are aromatic alcohols containing alcoholic hydroxyl group, or their derivatives containing methyl ( $\text{CH}_3$ ), methoxyl ( $\text{OCH}_3$ ) or carboxyl ( $\text{CO}_2\text{H}$ ) substituents. Fungal metabolites belonging to phenols, pyrocatechol, resorcinol, quinol, hydroxyquinol and pyrogallol groups have been isolated from various fungal species and are included in Table 16.2.

Among the polycyclic aromatic metabolites, a large number of bi- and tri-cyclic phenol derivatives are known to be synthesized by fungi. These belong to methylquinones (citrinin, atrovenetin, pulviloric acid etc.), benzophenone derivatives (sulochrin, dihydrogeodin etc.), spirans (geodin, geodoxin griseofulvin etc.), diphenyl ether, depsidone and Naphthalene derivatives (asteric acid, alternariol, xanthocillin-X, etc.). Structure and sources of a few of them have already been indicated in Table 16.2. While their biosynthesis has been discussed early in this chapter.

### Terpenes

Gibberellins are obviously among the most important terpenoid metabolites of fungi, on account of their growth, promoting effects on plant-shoot. The first compound of this group, viz. gibberellic acid (GA) was isolated by Yabuta (1935) from the cultures of the ascomycetous fungus *Gibberella fujikuroi*. Presently a total of 13 gibberellins are known, which according to their sequence of discovery are denoted as  $\text{GA}_1, \text{GA}_2, \text{GA}_3, \dots, \text{GA}_{13}$ . Of these, eight including,  $\text{GA}_1, \text{GA}_2, \text{GA}_3, \text{GA}_4, \text{GA}_7, \text{GA}_9, \text{GA}_{12}$  and  $\text{GA}_{13}$  have been isolated from *G. fujikuroi* (*Fusarium moniliforme*). Many of them have also, been isolated from higher plants. Gibberellins have stimulating effects on growth of shoot but not root; they can cause a reversal of genetie dwarfing and formation of flowering hormones. Biosynthesis of gibberellins by *Fusarium moniliforme* has been studied with labeled compounds (Birch *et al.* 1959). Gibberellins, being five-ring diterpenoids, it was suggested earlier that terpenes may act as important intermediates in their synthesis. Wenkert (1955) suggested that gibberellin could be derived from geranyl germiol in three steps shown below:

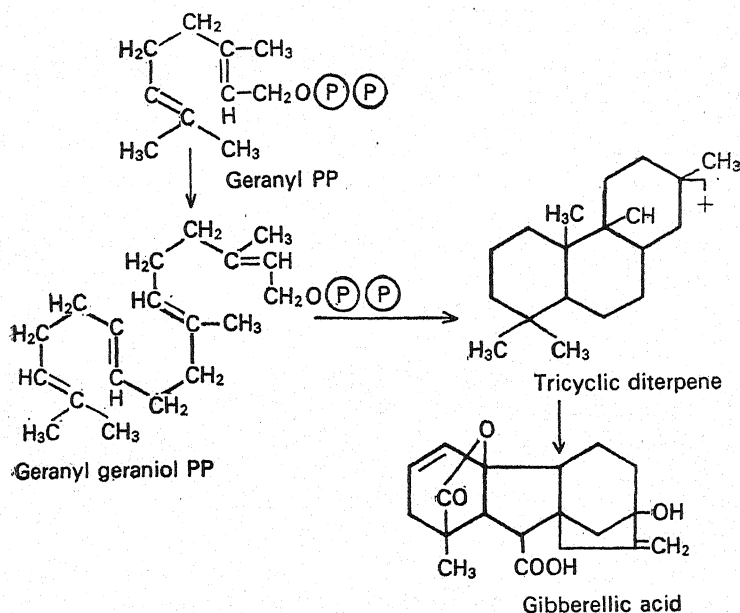


Fig. 16.8. Biosynthesis of gibberellic acid from geranyl PP.

Tracer experiments using labelled ( $2-^{14}\text{C}$ ) mevalonic acid and acetate ( $\text{CH}_3^{14}\text{COOH}$ ) have generally supported the above scheme (Birch *et al.* (1959). However, recently Gross *et al.* (1964) have shown that when *G. fujikuroi* was furnished with labelled (–)-Kaurene, it was incorporated intact into gibberellic acid, which has almost established the role of mycophenolic acid like precursors in the biosynthesis of gibberellins. The topic has been discussed further by Whalley (1967). Yet, at enzymic level much remains to be known about gibberellin synthesis.

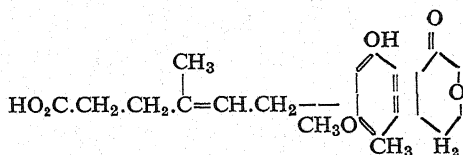


Fig. 16.9. Structure of mycophenolic acid.

### Tropolones

There are aromatic compounds characterized by seven membered

carbocyclic ring (Table 16.2) with characteristic double bond system. Fungal biosynthetic reactions leading to formation of tropolone ring is little understood and requires further investigation.

### Quinones and Derivative

A large variety of quinones and their derivatives are known to be produced by fungi. They essentially differ among themselves in the number of rings in their molecule as well as the manner in which they are linked. Otherwise, they are oxidized derivatives of quinols, the aromatic alcohols. Anthraquinones have received greater attention owing to their role in pigmentation of fungal mycelium. Sometimes they may comprise as much as 30% of the mycelial dry-weight. They usually contain three aromatic rings with one to five hydroxyl groups in their molecule, but several dianthraquinones are also known, which are formed as a result of carbon-to-carbon linkage of two similar molecules of anthraquinones. Two fungal dianthraquinones viz. skyrin and iridoskyrin are shown here, both of which are produced by *Penicillium islandicum* (Howard and Raistrick, 1954, Shibata *et al.* 1955) but the former is derived from two emodin units, while the latter from two islandicins:

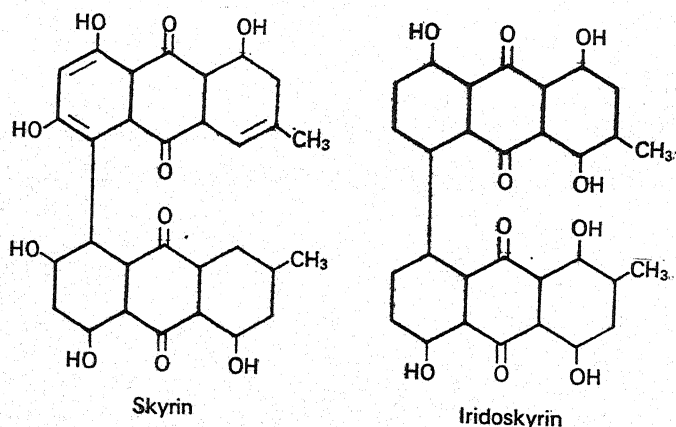


Fig. 16.10. Structure of skyrinky and ridosleyrin.

Representative types of other quinones synthesized by fungi have been shown in Table 16.2.

### Nitrogenous Metabolites

Fungi synthesize a variety of nitrogen containing metabolites,

ranging from ammonia and simple aliphatic amines like methylamine ( $\text{CH}_3\text{NH}_2$ ), ethylamine, n-propylamine etc., to oligopeptides and tricyclic nitrogen compounds. Besides, there are quarternary ammonium compounds like choline sulphate and acetylcholine; a number of amino acids and derivatives; nitro and Nitroso group containing metabolites; as well as various alkaloids and fungal poisons.

Ammonia seems to be cf. universal occurrence among fungi, while various aliphatic amines, which in many cases appear to arise by decarboxylation of corresponding amino acids, are also fairly distributed (Von Kamienski, 1958). Shibata *et al.* (1964) have enlisted eighteen different amines of fungal origin. In contrast, compounds with nitro and nitroso groups are scarce among fungi. Examples of such metabolites are  $\beta$ -nitropropionic acid ( $\text{O}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CO}_2\text{H}$ ) synthesized by *Aspergillus* spp. and *Penicillium atrovenerum* (Bush *et al.* 1951; Raistrick and Stossel, 1958) and p-methylnitrosamine benzaldehyde produced by *Clitocybe suaveolens* (Herrmann, 1960).

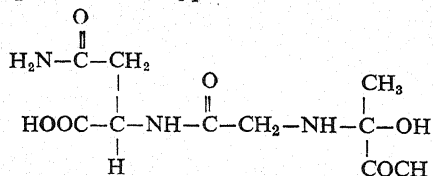
Various amino acids have been detected in fungi some of which are listed in Table 16.3.

TABLE 16.3

SHOWING SOME FUNGAL AMINO ACIDS

Source	Amino acids
1. <i>Neurospora crassa</i>	S—Methyl-L-Cysteine
2. <i>Neurospora crassa</i>	L—Carnitine
3. <i>Aspergillus oryzae</i>	L— $\alpha$ —Aminoadipic acid
4. <i>Aspergillus oryzae</i>	Stachydrine
5. <i>Polyporus sulphureus</i>	Homarine
6. <i>Polyporus sulphureus</i>	4-Imidazoylacetic acid
7. <i>Penicillium multicolor</i>	Pencolide
8. <i>P. frequentans</i>	N-Formylhydroxyaminoacetic acid (Hadacidin)
9. <i>Agaricus bisporus</i>	Agaritrine
10. <i>A. campestris</i> , <i>Polyporus sulphureus</i> and <i>Amanita muscaria</i>	Hercynine
11. <i>Claviceps purpurea</i>	Ergothioneine
12. <i>Coprinus micacens</i>	Allantonic acid
13. Yeast	$\beta$ -Methylanthionine

Oligopeptides comprise characteristic secondary metabolites of fungi, which may contain both peptide ( $-\text{CONH}-$ ) as well as the ester linkages. Thus, their chains or rings have in addition to amino acid molecules some nitrogen-free organic acids. In simpler forms like DL-fumarylalanine isolated from *Penicillium resticulosum*, there is only a peptide linkage between DL-alanine and one of the carboxylic groups of fumaric acid. Some oligopeptides produced by certain phytopathogenic fungi produce interesting effect upon the host plant which they attack and parasitize. *Fusarium* spp. have been found to produce several types of oligopeptides, like lycomarasmine, culmomasmine etc., which produce severe wilting effect upon their hosts and cause necroses at the tips and periphery of leaves. Lycomarasmine is a dipeptide and is supposed to have the following structure:



Two kinds of aspergillomarasmines are produced by *Aspergillus flavus-oryzae*:

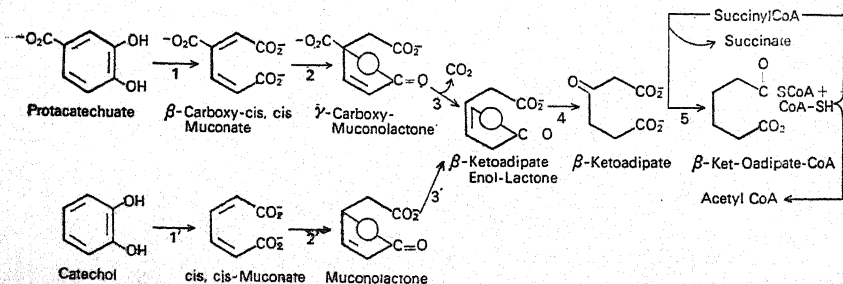
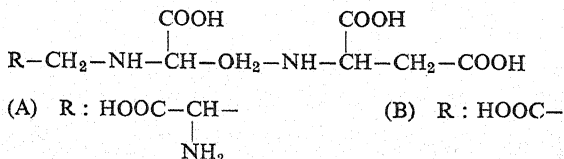
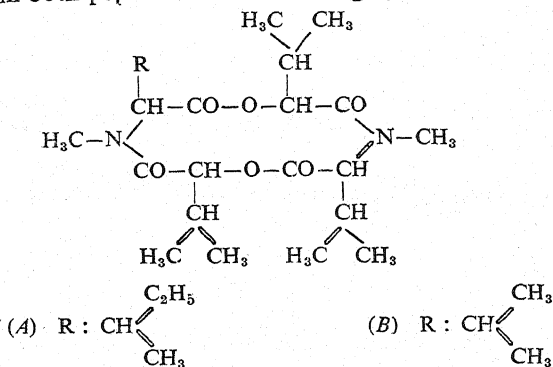


Fig. 16.11.

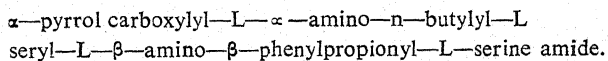
*Helminthosporium victoriae*, *H. Carbonum* and possibly *H. Sacchari* also produce polypeptide toxins (Luke and Gracen, 1972). Of these the toxin produced by *H. victoriae* is the best known and is composed

of a cyclic secondary amine ( $C_{17}H_{29}NO$ ), the victoxinine attached to a peptide, containing aspartic acid, glutamic acid, glycine, valine and leucine.

Peptide metabolites with antibiotic activities are also known from fungi. Several fusaria produce peptidal antibiotics (Cook *et al.* 1949), which have structures similar to Enniatin, the antibiotic active against *Mycobacterium tuberculosis*. These antibiotic metabolites contain both peptide and ester linkages, as shown below:



Peptide containing toxic metabolites are produced by *Penicillium islandicum*. Tatsuno *et al.* (1955) isolated a chlorine-containing peptide metabolite from the culture filtrate of this fungus, which has now been designated as cyclochlorotine (cf. Saito *et al.* 1971). Although the complete structure of this compound is yet to be elucidated, it appears to be a cyclic peptide containing serine,  $\alpha$ -amino-n-butylic acid,  $\beta$ -amino- $\beta$ -phenylpropionic acid and an unknown substance. Sato and Tatsuno (1968) have elucidated the amino acid sequence of the peptide, which is shown below, but the position of the two chlorine atoms is yet to be determined:



Structure of yet another toxic metabolite of *P. islandicum* viz. islanditoxin as proposed by Marumo (1955, 1959) is shown on the next page.

Sato and Tatsuno (1968) concluded that cyclochlorotine and islanditoxin were not identical. However, these peptides are quick acting hepatotoxin and causes the disappearance of glycogen granules in the injured liver, because it accelerates glycogen catabolism but inhibits its synthesis (Ueno *et al.* 1963). These toxic metabolites have received increasing attention not only due to their hepato-

toxic activity in man and animals, but also because they are synthesized by a fungus which in some places is one of the common storage fungi of cereals.

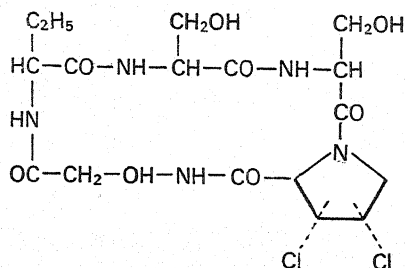


Fig. 16.12. Structure of Islanditoxin.

Any discussion on peptide metabolites of fungi would remain incomplete without mentioning the highly toxic peptide metabolites of *Amanita* spp. and some other Basidiomycetes. The deadly poisonous green mushroom *Amanita phalloides* called as the 'death cap' or 'deadly agaric', as well as some of its related species produce a number of toxic peptide metabolites, belonging to two main groups: (i) phallotoxins—mainly comprising phalloidin, phallacidin, phalloin, phallisin and phallin B and (ii) amatoxin—comprising  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\epsilon$ -amanitin and amanin. Majority of phallotoxins consist of a common cyclic heptapeptide skeleton, and yield upon hydrolysis six different amino acids. Amatoxins, on the other hand, are mostly composed of a cyclic octapeptide skeleton. The structural as well as other details have recently been discussed by Wieland and Wieland (1972). Interestingly, an antitoxin, known as antamanide and 100% active against phalloidin has recently been isolated from the extracts of the same fungus, viz. *A. phalloides* (T. Wieland *et al.* 1968 b, 1969 c). Moreover, this antitoxin is also a peptide consisting of a cyclic decapeptide of four different L-amino acids, viz. alanine, valine, phenylalanine and proline.

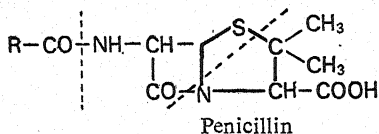
### Nitrogen Heterocyclic Compounds

A large number of nitrogenous heterocyclic metabolites including derivatives of pyrrols, pyrazine, pyridines, indole, azanthracene and phenoxazone compounds are known from fungi. Some of them are well known antibiotics, like penicillins, gliotoxin etc. others include toxic principles like fusaric acid, erythroskyrine, cyclopenin, cyclophenol,

etc., while still others are poisonous and hallucinogenic substances like psilocine, psilocybine etc. However, detailed discussion will be confined here to penicillins, and fusaric acid only.

*Penicillins.* Penicillins and their derivatives are known to be produced by several fungal organisms, of which species of *Penicillium* and *Aspergillus* are more common. *P. chrysogenum* series including *P. notatum* are particularly good sources of this antibiotic and are commercially harnessed for the same.

Extensive investigation on chemical structure, biosynthesis, antibiotic activity, etc. has amassed considerable data and the pertaining literature has often been reviewed (Clarke *et al.*, 1949; Abraham *et al.* 1949; Arnstein, 1957; Demain, 1959). Several kinds of penicillin are known, many of which have now been chemically synthesized. Naturally occurring penicillins have identical chemical skeleton mainly comprised of thiazolidine- $\beta$ -lactam structure derived from L-cystine and L-valine, besides substituted acetic acid and they differ among themselves only with respect to the side-chain (R):



In benzylpenicillin, which is used for medical purposes the side-chain contains phenylacetic acid. In other penicillins the side chain may contain a variety of groups including aromatic rings (as in Penicillin G) or aliphatic groups like H-6-aminopenicillic acid (as in Penicillin N, cephalosporin N), etc.

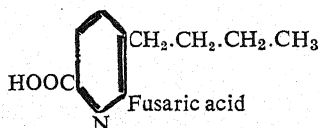
The biosynthesis of penicillins has been extensively studied with the use of isotopically labelled compounds. Arnstein *et al.* (1960) reported that when *Penicillium chrysogenum* was incubated with  $^{14}\text{C}$ -labelled valine, a tripeptide ( $\alpha$ -aminoadipyl-cysteinyl-valine) was obtained. Arnstein and Morris (1960) proposed that this tripeptide may be converted to penicillin N (cephalosporin N) by appropriate ring closures. This is amply supported by the observation that penicillin production is doubled when L-aminoadipic acid is exogenously supplied (Somerson *et al.* 1961). In the final stage, the  $\alpha$ -aminoadipoyl side chain of cephalosporin N is exchanged for a phenacyl group.



*Fusaric acid.* This metabolite was first reported in 1934 from *Fusarium heterosporum*, but its toxic nature was recognised about two decades later by Gaumann *et al.* in the year 1952, who also reported its occurrence from *F. oxysporum* f. *lycopersici*, *F. oxysporum* f. *vasinfectum* and *Gibberella fujikuroi*. Since then this phytotoxic metabolite has been detected in various *Fusaria* (Gaumann, 1957; Kalyansundaram, 1958; Heitefuss *et al.* 1960 a, b; Page, 1961; Trione, 1960 a, b; Prasad and Chaudhary, 1974).

Biosynthesis of fusaric acid is accomplished even in synthetic media, which shows that no additional nutrition is required for its production. However, it has been noticed that its production is conditioned by the amount of zinc present in the culture medium. Kalyanasudaram and Saraswathi Devi (1955) noted that secretion of fusaric acid by *F. oxysporum* f. *vasinfectum* required 0.08 to 0.4 ppm of zinc, the optimum concentration being 0.24 ppm. Prasad and Chaudhary (1974) also recorded stimulatory influence of zinc on fusaric acid production by *F. oxysporum* f. *udum*.

Fusaric acid, with empirical formula  $C_{10}H_{13}O_2N$ , is a pyridine-carboxylic acid and is chemically known as 5-butylpicolinic acid. It has the following chemical structure:



It is now well recognised that fusaric acid is produced during the rapid growth phase, and is not a product of autolysis. (Sandhu, 1960). Its synthesis seems to be linked with the intermediates of Krebs cycle and is a primary metabolite. Although growing hyphae secrete fusaric acid, most of it is liberated after mycelial autolysis starts (Stoll, 1954). This metabolite has also been detected in the mycelial extracts of different strains of *Fusarium oxysporum* (Starratt and Madhosingh, 1967, Prasad and Chaudhury, 1974), which indicates that the entire quantity synthesized by the fungus is not secreted out, rather some of it is retained in the hyphae.

Synthesis of fusaric acid *in vivo* has also been studied and some isotopic data were obtained to demonstrate its production in the tissue of the host-plant (Kern and Sanwal, 1954; Kern and Kluepfel, 1956). Direct detection of this metabolite in the tissue-extract of diseased host plants has also been attempted and met with appreci-

able success (Lakshminarayanan and Subramanian, 1955; Kalyanasundaram and Venkata Ram, 1956; Page, 1959 b). Kalyanasundaram (1958) detected fusaric acid in the rhizosphere soil of tomato-plant, secreted apparently by *Fusarium lycopersici*. In contrast, there are certain reports (Heitefuss *et al.* 1960 a), according to which no fusaric acid is produced in the host tissues, although the same pathogen produces fusaric acid in culture solution. They further concluded that this toxin had apparently no role in pathogenicity. Kuo and Scheffer (1964) have also doubted the role of fusaric acid in disease development, and full details are not clearly understood.

This metabolite is toxic to bacteria, algae, fungi and angiospermic plants, some of its notable effects are tabulated below:

TABLE 16.4

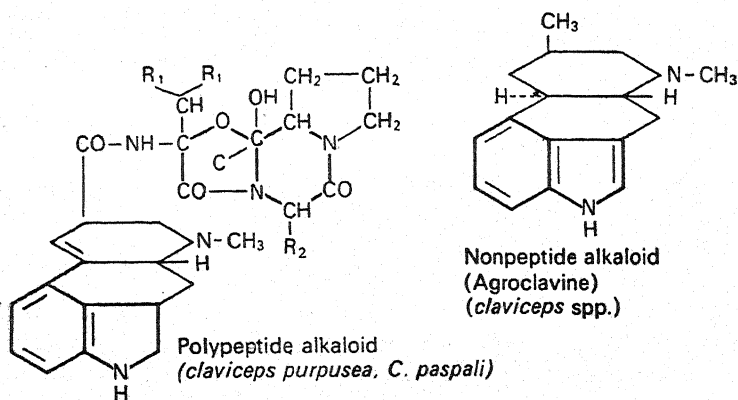
SHOWING TOXIC EFFECTS OF FUSARIC ACID ON VARIOUS PLANTS

Organisms	Effect	Concentration
Bacteria	growth inhibited	$10^{-4}$ to $10^{-3}$ M
Green algae		
<i>Spirogyra nitida</i>	Permeability affected	$5 \times 10^{-8}$ M
<i>Ustilago maydis</i>	Germination of brandspores	$1.5 \times 10^{-4}$ M
Rye, maize and pea plants	injury caused	1,000 to 2,000 mg/kg fresh weight
Tomato plants	Do	150 mg/kg fresh weight
Cotton plants	Do	10 to 20 mg/kg fresh weight

However, whether fusaric acid is responsible for causing all the symptoms in the diseased host plant infected with wilt-fusaria is yet to be established.

### Miscellaneous Compounds

A number of alkaloids, steroids, terpenoids and other complex compounds have also been recorded from different fungi, but detailed discussion about them is not possible within the present scope. Shibata *et al.* (1964) have enlisted a large number of such metabolites alongwith their sources and relevant literature. Ergot-alkaloids from *Claviceps purpurea* and some related species are well known fungal products, some of which are shown below alongwith their sources;



Some fungal metabolites are known to contain unusual radicals like arsenic, chlorine, etc. Some chlorine containing metabolites, like geodin, griseofulvin etc. have already been discussed. Other chlorinated metabolites of fungi include (i) drosophilin or p-methoxy-tetrachlorophenol, synthesized by *Drosophila subatrata* (Syn. *Psathyrella subatrata*); (ii) nalgoloxin, a chlorinated anthraquinone synthesized by *Penicillium nalgiovensis*; (iii) caldariomycin, a cyclopentane with two chlorine atoms, synthesized by *Caldariomyces fumago*; etc.

Some fungi incorporate available inorganic radicals like arsenic (As), selenium (Se) and tellurium (Te) also in organic combinations. A toxic arsenic containing gas, trimethylarsine,  $(CH_3)_3As$ , is produced by *Scopulariopsis brevicaulis* (= *Penicillium brevicaulis*), when provided with arsenic compounds (Challenger *et al.* 1933). Similarly,  $(CH_3)_2Se$  (dimethyl selenide) and  $(CH_3)_2Te$  (dimethyl telluride) are produced when these elements are available to the fungus.

To summarise briefly, the various structural components of fungal metabolites are derived as follows:

(i) Majority of the phenolic rings are derived from acetate units, although a few of them may have shikimic acid or its biogenetic associates as their precursors.

(ii) The various additional carbon atoms such as C-methyl, O-methyl, and N-methyl groups, which are present as substituents to the major structural components, are derived frequently from formate or its biological equivalent in the  $C_1$  metabolic pool. In some cases, C-methyl groups are derived from C-3 of propionic acid.

(iii) Terpenes, steroids and terpenoid residues attached to phenolic rings have mevalonic lactone as their immediate precursor and, therefore, may be considered as indirect derivatives of acetate.

(iv) Some of the structural constituents have amino acids as their precursors.

### **Significance of Aromatic Fungal Metabolites**

From the foregoing discussion it is evident that fungi produce a large variety of aromatic compounds with a majority of them as products of secondary metabolism. The primary cyclic metabolites are largely represented by simple or substituted phenols, many of which are utilized by fungi as sources of carbon and energy, and convert them into aliphatic cellular components through specific metabolic pathway. One of such pathway, viz. the  $\beta$ -ketoadipate pathway and its various steps have already been described in Chapter VI. The possible roles of the secondary aromatic metabolites on the contrary, have been a point of discussion and, therefore, the same deserves a brief account.

Foster (1949) considered that the aromatic metabolites of fungi are the products of metabolic shunts. Our knowledge to-date also indicates that most of them do not belong to the products of principal metabolic pathways, rather they represent some kind of metabolic derailment or overflow etc. Sometimes environmental and/or nutritional factors are also associated with secondary metabolism, because many of the secondary metabolites have been obtained from growth on routine culture media, which may not be properly balanced for specific organisms to carry on their normal metabolic activities.

The secondary metabolites in general have been assigned three kinds of functions, viz. (i) general function, (ii) specialised functions of a few of them affecting the producing organism itself, and (iii) specialised functions of a few specific metabolites affecting other organisms.

(i) *General functions.* Various roles have been assigned to the secondary metabolites performing general functions. They might be serving either as (i) waste product of cellular metabolism, (ii) reserve-food storage substances, (iii) break-down products of cellular macromolecules, or as (iv) safety-valve shunts of very low molecular-weight precursors into harmless product. However, only the last function

proposed by Woodruff (1966) has received some support (Weinberg, 1970), although on the basis of some indirect evidences only (Leonard *et al.*, 1958; Steenbergen *et al.*, 1969; Weinberg and Goodnight, 1969). According to this concept, it is not the metabolites which are significant to the organism, but the secondary metabolic processes which produce them are more important for the organisms. It is argued that when microbial cells enter the phase of non-balanced growth, they are destined to die of very high accumulation of precursors like acetate, malonate, pyruvate, specific amino acids or purine nucleotides, in spite of the various metabolic regulatory mechanisms operating in them. But this danger is warded-off by the secondary metabolic processes, which set in and channelise these precursors into the formation of harmless products. Obviously, utilization of the precursors is important, which is achieved through secondary metabolism, and hence secondary metabolites may simply be considered as the by-products which may not have any specific function in the physiology of the producing organisms. The other three proposals have been questioned, because secondary metabolites (i) do not accumulate either during balanced growth phase or late stationary phase, (ii) many of them are excreted out and do not undergo further metabolism in the producing cell; and (iii) they are known to be formed *de novo*, after the primary biosynthetic processes have stopped.

(ii) *Special functional affecting the producing cells.* Some secondary metabolites have been assigned functions that would affect the producing cell itself. It has been proposed that certain antibiotic substances are often produced in small amounts by the growing cells to inhibit macromolecular synthesis or function (Yoshida *et al.*, 1966). Sensitivity of growing cells to the same kind of antibiotic principle, which they produce later on, has also been shown, but fungal antibiotic penicillin is an exception of such a behaviour, Piricularin, a vivotoxin produced by rice-blast fungus, *Piricularia oryzae*, is more toxic to the fungus itself than to the host plants, and the fungus produces a specific protein to inactivate the antifungal property of the toxin, but not the phytotoxic property.

Secondary metabolites like hispidin synthesized by *Polyporus hispidus* and *P. schweinitzii*, and converted into lignin like polymers in the ripening fruiting-bodies, as well as polymers of perylenequinone found in ascomycetous fruits, perform structural functions (Bu'Lock, 1967). Many other quinones and phenolic derivatives might also be serving in similar capacity.

Weinberg (1957) suggested that secondary metabolites may also serve as strong metal-binding agents. However, adequate evidence, particularly in fungi, is very much lacking to support this contention. Similar is the case with the idea that secondary metabolites are simply overproduced components of walls of vegetative cells.

(iii) *Specialized functions affecting other organisms.* According to earlier concept, it was believed that synthesis of such kinds of secondary metabolites, which are poisonous or toxic to the neighbouring organisms, confers a selective advantage to the producing organisms. However, observations do not indicate such advantageous survival position for the toxinogenic organisms in nature. Moreover, in several cases the toxic principle is produced only under specific laboratory conditions. Even where the toxinogenic organisms do produce such substances in nature, the metabolites generally render the environment unsuitable for the producing organisms themselves. Thus, it is difficult to understand how the fungal cells producing different mycotoxins, hallucinogenic compounds etc. can derive any benefit from these metabolites acting upon a plant or animal cells.



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